REMARKS

In the Office Action dated June 15, 2009, claims 45-46, 50-54, 60-61, 63-65, 68-71, 87-89, and 91 were pending. Claims 63 and 91 were withdrawn from consideration as allegedly directed to a non-elected invention. Claims 45-46, 50-54, 60-61, 64-65, 68-71 and 87-89 were under consideration and were rejected under 35 U.S.C. §103(a) as allegedly obvious.

This Response addresses each of the Examiner's rejections. Applicant therefore respectfully submits that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

Substance of Telephone Interview

A telephone interview was conducted between the undersigned attorney, Examiner Sgagias, and Supervisory Examiner Falk on May 5, 2009. The rejections raised in the Final Action dated September 30, 2008 were discussed in light of Applicant's Response filed on March 30, 2009. Examiner Sgagias has provided a summary of the interview, which is attached to the outstanding Office Action.

Applicant, through the undersigned attorney, wishes to thank both Examiners for the courtesy extended to Applicant during the interview.

The interview summary was correct in stating that the Examiners agreed to withdraw the §102(b) rejection and to re-examine the §103(a) rejection raised in the Final Action. However, the interview summary was incorrect in stating that the undersigned attorney proposed an amendment to claim 45 to recite "wherein said embryonic cell is an endodermal or ectodermal cell *and not a vascular endothelial cell.*" Such mischaracterization

appears to reflect a continued misunderstanding of the invention by Examiner Sgagias. Hence, the undersigned attorney submits the following for clarification.

During the interview, Examiner Sgagias questioned the claim language "embryonic cell", which appears on line 3 of claim 45, and which also appears in the expression "wherein said embryonic cell is an endodermal or ectodermal cell", introduced in Applicant's Response dated March 30, 2009. Examiner Sgagias contended that the phrase "embryonic cell" was unclear and could be interpreted as referring to or encompassing the "human embryonic (hES) stem cell" recited earlier in the claim, because the hES cell was also an embryonic cell.

The undersigned attorney stated during the interview that Examiner Sgagias' interpretation was incorrect; that two cell sources are involved in the co-culture as claimed: (1) an undifferentiated human embryonic (hES) stem cell, and (2) an embryonic cell or extracellular medium thereof; that "said embryonic cell" could not, and should not, be interpreted to refer to the "human embryonic (hES) stem cell", because the proper reference to the hES cell would have been "said hES cell" or "said human embryonic stem cell". Because the Examiners agreed to this clarification from the undersigned attorney, and because Examiner Sgagias admitted that the rejections in the Final Action were raised, at least in part, based on an incorrect interpretation of the claim language, the Examiners agreed to withdraw the anticipation rejection and reconsider the obviousness rejection.

Further, because the term "embryonic cell", properly interpreted, refers to the cell used in the co-culture with an hES cell, rather than the hES cell itself, and because a vascular endothelial cell is a cell which the hES cell differentiates to as a result of the co-culture, it is apparent that the expression, "wherein said embryonic cell is an endodermal or

ectodermal cell *and not a vascular endothelial cell*" (as stated by the Examiner in the interview summary), is illogical.

Claim Amendments

The instant claim listing has corrected the status indicator of claims 63 and 91 as "withdrawn". Applicant acknowledges that the claims are examined to the extent the hES cell is differentiated to a cardiomyocyte and not a vascular endothelial cell, and that the hES cell is cultured in the presence of an endodermal or ectodermal cell.

Rejection Under 35 U.S.C. §103

Claims 45-46, 50-54, 60, 64-65, 68-71 and 87-89 are rejected under 35 U.S.C. §103(a) as allegedly obvious over Reubinoff et al. (Nature Biotechnology, 18: 399-404, 2000) in view of van den Eijnden-van Raaij et al. (Mechanisms of Development, 33: 157-166, 1991), Skerjanc (Trends Cardiovasc Med 1999; 9:139-143, 1999), and Itskovitz-Eldor et al. (WO 00/70021).

Applicant respectfully disagrees with the Examiner's rejection. Applicant will first identify the deficiencies of the references individually, and then discuss the inappropriateness in the Examiner's combination of the references.

Reubinoff

According to the Examiner, Reubinoff teaches a method for the derivation of cardiomyocytes from the hES2 cells. In referring to page 401, 2nd col., 2nd paragraph of Reubinoff, the Examiner alleges that beating cardiomyocytes were observed following co-culturing of the hES2 cells with mouse embryonic fibroblasts (MEFs). The Examiner further

states that Reubinoff differs from the present invention for not teaching co-culture with endothelial cells (END2).

In the first instance, Applicant notes that the Examiner refers to co-culture with endothelial cells. Applicant respectfully draws the Examiner's attention that the co-culture of the present invention is between hES cells and embryonic cells; and preferably, the embryonic cells are embryonic endodermal cells, and more preferably END-2 cells which are endodermal cells. Endodermal and endothelial cells are distinct cells.

Further, the passage in Reubinoff (page 401, 2nd col., 2nd paragraph) which is referenced by the Examiner states:

> *"Contracting muscle was also seen infrequently in these cultures. Although contracting muscle was a rare finding, we often observed bundles of cells that were stained positively with antibodies directed against muscle-specific forms of actin, less commonly cells containing desmin intermediate filaments."*

This paragraph indicates that the derivation of contracting muscle cells is an unreliable art and more importantly in the methods described in Reubinoff the finding of the cell type is "rare". Hence, those skilled in the art would not consider the Reubinoff reference to provide a reliable method for the derivation of cardiomyocytes from hES cells. Indeed, it is respectfully submitted that the description for the methods and materials in Reubinoff would not enable a person skilled in the art to consistently obtain cardiomyocytes.

Furthermore, Reubinoff does not teach any co-culture of the hES cells with an embryonic cell for purposes of differentiation into cardiomyocytes specifically. While Reubinoff does teach co-culturing hES cells with a fibroblast layer, the fibroblast layer is provided for the purpose of maintaining the hES cells in an undifferentiated state. See, e.g., page 399, right column, last two lines of Reubinoff. Hence, Reubinoff teaches away from the

invention where the embryonic cells used in the co-culture *induce* the hES cells into differentiation.

The Examiner also apparently makes the assumption that the contracting muscle in Reubinoff is a cardiomyocyte. However, there is no support for this assumption in Reubinoff. The cells could also be spontaneously contracting smooth muscle cells that would also express the identified marker profile and contracting phenotype.

Hence, Applicant respectfully submits that Reubinoff does not provide adequate teaching for derivation of cardiomyocytes from hES cells as the Examiner has alleged, much less derivation based on co-culture with embryonic cells.

van den Eijnden-van Raaij and *Skerjanc*

The Examiner' reliance upon van den Eijnden-van Raaij and Skerjanc is also flawed. These articles solely disclose the use of the mouse P19 embryonic carcinoma cells (EC cells)[1]. The present claims are directed to the use of human ES (hES) cells as opposed to any other pluripotent type of cells such as mouse embryonic carcinoma (EC) cells. There are significant distinctions between mouse EC and human ES cells, and consequently significant distinctions between their behaviors. Hence, those skilled in the art would not reasonably expect the teachings of van den Eijnden-van Raaij and Skerjanc, which relate to mouse EC cells, to apply to human ES cells.

Mouse EC cells are derived from malignant teratocarcinomas. They are generally karyotypically abnormal (aneuploid); in other words, they do not carry a normal complement of chromosomes. Applicant directs the Examiner's attention to an abbreviated

[1] It is noted that on page 4 of the Action, the Examiner makes reference to "mESC" in Figure 1 on page 158 of van den Eijnden-van Raaij. There is no reference to "mESC" in the description of Figure 1 or anywhere on page 158. Similarly, on page 4 of the Action, the Examiner makes reference to "ES cells" on page 159, first column of van den Eijnden-van Raaij. The identified portion of van den Eijnden-van Raaij only refers to mouse EC p19 cells.

copy of *Appendix C, C-8, Stem Cells: Scientific progress and future research directions – NIH publication June 2001 http:\\stemcells.nih.gov/info/scireport/appendixC.asp* (attached hereto as **Exhibit 1**), which discusses the properties of EC cells and highlights the distinctions between human ES cells and EC cells (including mouse EC and human EC cells). The EC cells are adapted for tumor growth and when differentiated, show an inability to differentiate into well-recognized cell types. Even when transplanted, these cells retain the ability to form teratocarcinomas. EC cells are generally known as the *malignant* version of ES cells or cells of the inner cell mass (ICM), and generally do not differentiate significantly to a diversity of cell types (see *Andrews, P.(2002) Phil. Trans R. Soc. Lond. B, 357, 405 – 417,* attached hereto as **Exhibit 2**).

Conversely, ES cells from different species are considered "genetically normal" as are the differentiated cell types that arise from them. ES cells are derived from the ICM of blastocyst stage embryos. Since they are karyotypically normal, when grown in immunodeficient mice, they form well-defined non-malignant teratomas with well-organized tissues representing all the three germ layers indicative of pluripotentiality (*Thomson et al 1998 referenced in Andrews, P (2002)*). In addition, when mouse ES cells are injected into a mouse blastocyst and the blastocyst is returned to the uterus, chimeric mice are formed in which most, if not all tissues (including the germ cells), can contain progeny of the donor mouse ES cells.

In contrast, mouse EC cells rarely contribute to many cells types in the chimera and additionally, rarely if ever contribute to the germ tissue as do the mouse ES cells. The ability of mouse ES cells to contribute so widely to different cell types either in teratomas

or chimeras is a defining characteristic of mouse ES cells that demonstrates their true pluripotent nature (*Andrews, supra,* as **Exhibit 2**).

Notably many mouse and human EC cells have limited differentiation capacity or have completely lost their ability to differentiate and have become "nullipotent" (*Andrews et al., Biochemical Society Transactions 33, part 6, 1526-1530, 2005,* attached hereto as **Exhibit 3**, especially page 1527, right column, bottom paragraph). Therefore, even human EC cells do not behave in the same manner as human ES cells and have a very restrictive differentiation capacity.

Mouse ES and EC cells will generate muscle and mesodermal derivatives whereas human EC cells generally are not capable of mesodermal differentiation (*Draper, J.S. et al 2002 J. Anat. 200, 249 – 258*) (attached hereto as **Exhibit 4**). Human EC cells also commonly form the trophoblastic lineage while this does not occur with mouse EC cells (*Andrews et al. (2005), supra,* **Exhibit 3**).

The human EC cell line NTERA-2, one of the few human EC cell lines that is able to differentiate, is not capable of mesodermal differentiation and shows no evidence of cardiomyocyte differentiation (*Gokhale et al. 2000 Cell Growth Diff, 11, 157 – 162, referred to in Draper et al. (2002) (**Exhibit 4**) on page 256, left column, bottom of middle paragraph)*; whereas as shown in Mummery *et al.* (2002), *Circulation* 107: 2733 – 2740 (attached hereto as **Exhibit 5**), hES cells are capable of being differentiated in a controlled and reproducible manner into cells of the mesodermal lineage and to form the cardiomyocytes (see abstract, for example).

The response of the mouse EC P19 cells to differentiation agents that enhance cardiomyocyte differentiation also differs from that of hES cells. The teaching of Skerjanc

(cited by the examiner) highlights the required presence of 0.5-1% DMSO (dimethylsulfoxide) to induce cardiomyocyte formation from mouse EC P19 cells as aggregates in suspension culture. Importantly, DMSO has no effect on the differentiation of hES cells to the cardiomyocyte lineage (see *Xu et al., Circulation Research 2002; 91: 501-508*, attached hereto as **Exhibit 6**; see, e.g., the abstract). Therefore, it would not have been obvious that a differentiation inducing signal would have the same effect across both species and also cell type (e.g., mouse or human, EC versus ES cells). It is also notable that DMSO treatment of human EC cells fails to be an effective inducer of differentiation of human EC cells (see *Draper et al. (2002)* (**Exhibit 4**), page 254).

There are also prominent differences in the expression of cell surface markers, such as SSEA1, which is expressed on mouse EC and ES cells but not on human EC or ES cells. Human EC and ES cells also express glycolipids SSEA3 and SSEA4, proteoglycan antigens TRA 1-60, TRA 1-80 and GCTM-2 and protein antigens Thy1 and MHC class 1, all of which are not seen on mouse EC or ES cells (*Andrews et al. (2005), supra,* **Exhibit 3**).

Accordingly, the physical characteristics and the differentiation profiles of these cell types (mouse EC and human ES cells) are quite different. Observations made with one cell type cannot be extrapolated to another. In fact, the uncontrolled nature of mouse EC cells, particularly in their underlying karyotypic instability and response to differentiation agents make their predictability difficult in comparison to other karyotypically normal cell types such as human ES cells. Even human EC cell types are not able to respond to differentiating factors in a manner that is similar to hES cells, even though human EC cells can be differentiated to some lineages to a limited degree.

There is no correlation between the experiments performed in mouse EC cells with that performed in hES cells. When considering the abnormal nature of a mouse EC cell, one skilled in the art would have had no reasonable expectation that conditions applied to mouse EC cells would apply to or operate with hES cells at all. This is because hES cells, although still regarded as a tissue culture artefact (*Zwaka T.P. and Thomson J.A 2005 Development, 132, 227 – 233)* (attached hereto as **Exhibit 7**), are far more representative of a normal pluripotent cell type than a mouse EC cell.

For all the above reasons, it is respectfully submitted that the generation of cardiomyocytes from hES cells, which has been demonstrated for the first time by the present invention, was entirely unexpected and therefore unobvious over van den Eijnden-van Raaij and Skerjanc, both of which are directed to mouse EC cells.

Itskovitz-Eldor (WO 00/70021)

The deficiencies of Reubinoff, van den Eijnden-van Raaij and Skerjanc are not cured by combining with Itskovitz-Eldor (WO 00/70021). WO 00/70021 is the publication of a patent application which is believed to arise from the Itskovitz-Eldor article (*Molecular Medicine*, 6(2): 88-95, 2000), which was relied on by the Examiner in the previous Action.

Similar to the Itskovitz-Eldor article (*Molecular Medicine*, 6(2): 88-95, 2000) Itskovitz-Eldor (WO 00/70021) does not describe any co-culture of hES cells with an embryonic cell to generate cardiomyocytes. In fact, in both Itskovitz-Eldor citations, the hES cells are removed from the fibroblast feeder layer prior to differentiation to embryoid bodies (EB) (see pages 5-6, "Formation of human cystic EBs", WO 00/70021). It is noted that the Examiner has apparently recognized the deficiency of Itskovitz-Eldor article (*Molecular*

Medicine, 6(2): 88-95, 2000) and has withdrawn the §102 rejection based on Itskovitz-Eldor article (*Molecular Medicine*, 6(2): 88-95, 2000).

Combination of references improper

Hence none of the citations raised by the Examiner to support this §103 objection, alone or in combination, teach or suggest the claimed method involving co-culture of hES cells and an embryonic cell. The only example of co-culture is found in van den Eijnden-van Raaij and Skerjanc; yet both of these citations refer to mouse EC p19 cells. As discussed above, because of the substantial distinctions between hES cells (instant invention) and mouse EC cells, those skilled in the art would not have had any reasonable expectation of success in applying the teachings and observations of mouse EC cells, including co-culturing a mouse EC cell with an END-cell, to human ES cells.

Further, there is no reason to combine either Reubinoff or Itskovitz-Eldor with van den Eijnden-van Raaij or Skerjanc, because neither Reubinoff nor Itskovitz-Eldor teaches co-culture of hES cells with embryonic cells for the purpose of inducing differentiation. In fact, there is no recognition in either Reubinoff or Itskovitz-Eldor that the co-culture with the embryonic cells is crucial in the differentiation process of hES cells to cardiomyocytes. In Reubinoff, the use of a fibroblast feeder layer (a potential co-cultured embryonic cell) is specifically taught to be used for maintaining the cells in an undifferentiated state. The contracting muscle cells observed by Reubinoff arise because the fibroblast feeder layers can no longer sustain or maintain the hES cells in an undifferentiated state after prolonged culture and spontaneous differentiation has occurred as a result of the hES cells not being transferred onto fresh fibroblast feeder cells. In Itskovitz-Eldor, the cells are removed from the feeder

layer to allow for subsequent differentiation. Clearly, neither Reubinoff nor Itskovitz-Eldor teaches toward the use of a co-cultured cell for inducing differentiation of hES cells.

Therefore, Applicant respectfully submits that the presently claimed invention, as a whole, is not obvious over the combination of Reubinoff in view of van den Eijnden-van Raaij et al., Skerjanc, and Itskovitz-Eldor. Withdrawal of the rejection under 35 U.S.C. §103(a) is respectfully requested.

Claim 61 is rejected under 35 U.S.C. §103(a) over Reubinoff in view of van den Eijnden-van Raaij et al., Skerjanc, and Itskovitz-Eldor, and further in view of Carpenter et al. (US 20020081724A1).

It is respectfully submitted that because claims 45 and 46 are not obvious over Reubinoff in view of van den Eijnden-van Raaij et al., Skerjanc, and Itskovitz-Eldor, and because Carpenter et al. do not cure the deficiencies of these references, claim 61, which is ultimately dependent on claims 45 and 46, is also unobvious. Withdrawal of the rejection of claim 61 is respectfully requested.

Conclusion

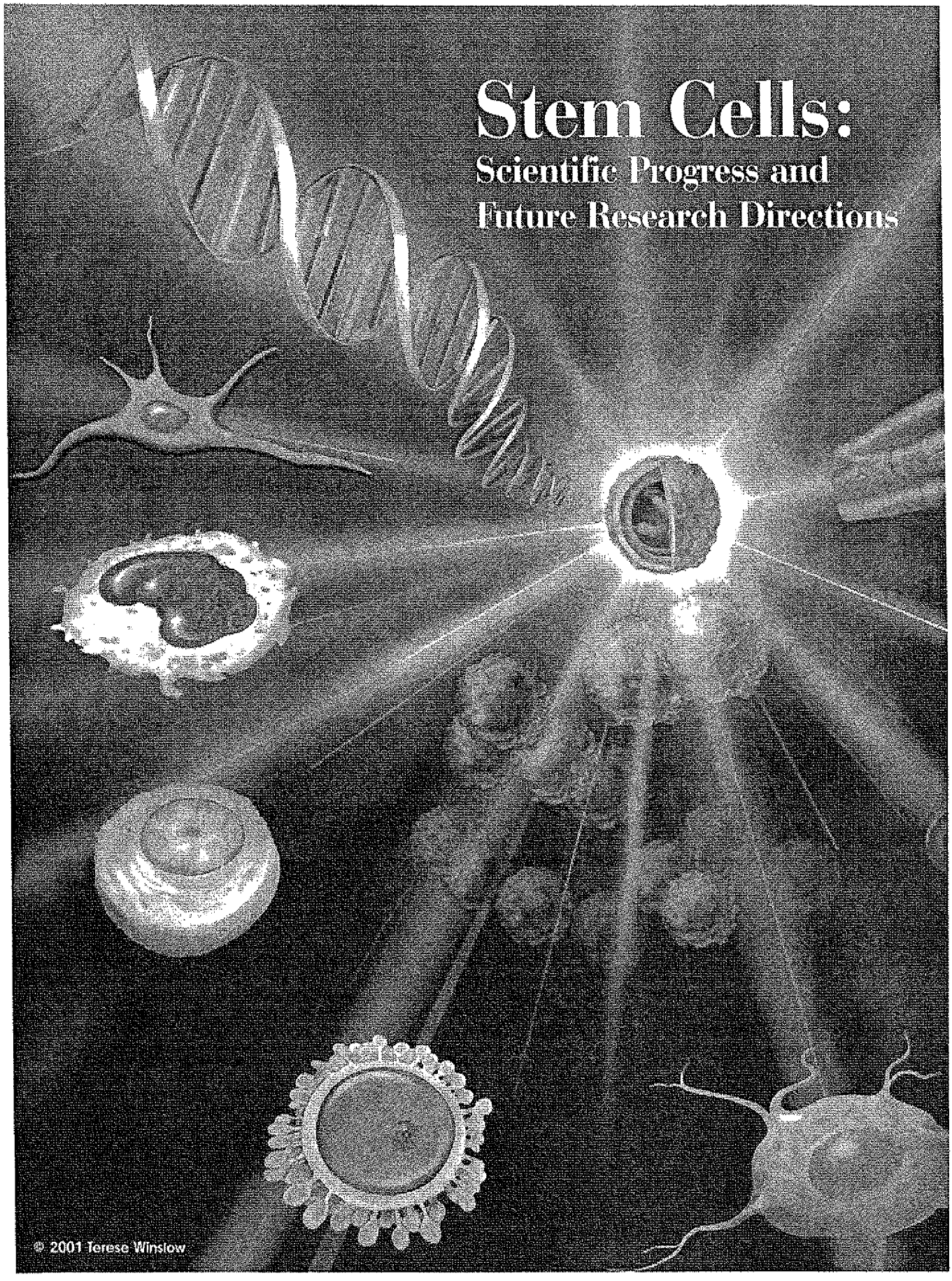
In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

Xiaochun Zhu
Registration No. 56,311

Scully, Scott, Murphy & Presser, P. C.
400 Garden City Plaza-STE 300
Garden City, New York 11530
Telephone: 516-742-4343 (XZ:ab/dk)
Enc.: Exhibits 1-7.

EXHIBIT 1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STEM CELLS: SCIENTIFIC PROGRESS AND FUTURE RESEARCH DIRECTIONS

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human ES and EC cells express MHC Class I antigens, which are responsible for immunogenicity (see Chapter 6, Autoimmune Diseases and the Promise of Stem Cell-Based Therapies). Like mouse ES and EC cells, undifferentiated human ES and EC cells strongly express the transcription factor Oct-4 [26, 4], which is widely regarded as a hallmark of pluripotent embryonic cells [20, 28, 29] (see Table C.1, Comparison of Mouse, Monkey, and Human Pluripotent Stem Cells).

Human ES and EC cells differ in important ways. Human ES cells are euploid, meaning they carry the normal complement of chromosomes. In contrast, human EC cells are aneuploid; their chromosomes are distinctly abnormal. (Interestingly, the chromosomes in mouse EC cells do not appear as abnormal, although they do carry subtle chromosomal abnormalities.) The ability of both cell types to differentiate into various tissue types has been explored by injecting human ES and EC cells into

Table C.1. Comparison of Mouse, Monkey, and Human Pluripotent Stem Cells

Marker Name	Mouse EC/ ES/EG cells	Monkey ES cells	Human ES cells	Human EG cells	Human EC cells
SSEA-1	+	-	-	+	-
SSEA-3	-	+	+	+	+
SSEA-4	-	+	+	+	+
TRA-1-60	-	+	+	+	+
TRA-1-81	-	+	+	+	+
Alkaline phosphatase	+	+	+	+	+
Oct-4	+	+	+	Unknown	+
Telomerase activity	+ ES, EC	Unknown	+	Unknown	+
Feeder-cell dependent	ES, EG, some EC	Yes	Yes	Yes	Some; relatively low clonal efficiency
Factors which aid in stem cell self-renewal	LIF and other factors that act through gp130 receptor and can substitute for feeder layer	Co-culture with feeder cells; other promoting factors have not been identified	Feeder cells + serum; feeder layer + serum-free medium + bFGF	LIF, bFGF, forskolin	Unknown; low proliferative capacity
Growth characteristics *in vitro*	Form tight, rounded, multi-layer clumps; can form EBs	Form flat, loose aggregates; can form EBs	Form flat, loose aggregates; can form EBs	Form rounded, multi-layer clumps; can form EBs	Form flat, loose aggregates; can form EBs
Teratoma formation *in vivo*	+	+	+	-	+
Chimera formation	+	Unknown	+	-	-

KEY

ES cell = Embryonic stem cell
EG cell = Embryonic germ cell
EC cell = Embryonal carcinoma cell
SSEA = Stage-specific embryonic antigen
TRA = Tumor rejection antigen-1
LIF = Leukemia inhibitory factor
bFGF = Basic fibroblast growth factor
EB = Embryoid bodies

immunocompromised mice. Injected human ES cells will form embryonic stem cell teratomas in mice, and the tumors consist of cells derived from all three primary germ layers [36]. In contrast, human EC cell lines vary in their ability to differentiate *in vivo*, but in general are more limited than are ES cells. For example, NTERA2 cl.D1 cells (which are derived from human TERA2 EC cells) generate only a few kinds of tissues, including primitive gut-like structures, and neural tissue after injection into immunocompromised mice [2].

The *in vitro* growth characteristics of human ES and EC cells are also being compared. Both cell types grow well in serum-containing medium on feeder layers of mouse embryonic fibroblasts that have been treated to block their proliferation. It is difficult to induce human ES cells to proliferate in the absence of feeder layers, unless conditioned medium from feeder cells cultures is added. However, many human EC cells lines, such as the NTERA2 line, are not dependent on feeder layers [2].

If human ES cells are removed from their feeder layers, they differentiate spontaneously into many cell types. Mouse ES cells, after removal from feeder layers, can be stimulated to divide and prevented from differentiating by adding LIF (leukemia inhibitory factor); neither human ES nor EC cells show this response to LIF. Instead, if human ES cells grow to confluence (where the cells grow to completely cover the culture plates), the cells aggregate and begin to differentiate spontaneously [26, 35]. Also, human ES cells grown in suspension cultures at high density will form embryoid bodies. Embryoid bodies are clumps or groupings of cells that form when cultured in plates or media and do not occur in nature. Embryoid bodies contain undifferentiated and partially differentiated cells [14]. However, human EC cells remain undifferentiated when grown at high density [4]. Whether these apparent differences in the *in vitro* growth characteristics of human ES and EC cells are meaningful or real is subject to debate [5].

The pluripotency of human EC cells does not equal that of human ES cells. Human ES cells can differentiate into a wide range of cell types *in vitro*, and can form teratomas with many cell types after injection into immune-deficient mice. The differentiation potential of most lines of human EC cells is more limited, both *in vitro* and *in vivo*. One human EC cell line, however, TERA2, differentiates easily *in vitro*. The well-studied morphogen, retinoic acid, induces TERA2 cells (and the subline NTERA2) to differentiate into neural precursors, which can then become mature neurons [4]. But when human ES cells are exposed to retinoic acid, they differentiate into a wider array of cell types than do human EC cells. As yet, it is not clear how the mechanism of action of retinoic acid differs in human ES cells versus human EC cells. It may be that, because of their tumor origin, human EC cells carry genetic variations linked to tumorigenesis that restrict their capacity for differentiation [5].

Thus, the *in vitro* and *in vivo* characteristics of human EC cells resembles that of human ES cells in certain respects, but not in others. Although ES cells will likely prove to be a better model for understanding human development than will EC cells [27], there may be some aspects of development that EC cells will reveal that ES cells will not [5].

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EXHIBIT 2

EC, ES → pluripotent

THE ROYAL SOCIETY

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From teratocarcinomas to embryonic stem cells

Peter W. Andrews

Department of Biomedical Science, Western Bank, University of Sheffield, Sheffield S10 2TN, UK
(*p.w.andrews@sheffield.ac.uk*)

The recent derivation of human embryonic stem (ES) cell lines, together with results suggesting an unexpected degree of plasticity in later, seemingly more restricted, stem cells (so-called adult stem cells), have combined to focus attention on new opportunities for regenerative medicine, as well as for understanding basic aspects of embryonic development and diseases such as cancer. Many of the ideas that are now discussed have a long history and much has been underpinned by the earlier studies of teratocarcinomas, and their embryonal carcinoma (EC) stem cells, which present a malignant surrogate for the normal stem cells of the early embryo. Nevertheless, although the potential of EC and ES cells to differentiate into a wide range of tissues is now well attested, little is understood of the key regulatory mechanisms that control their differentiation. Apart from the intrinsic biological interest in elucidating these mechanisms, a clear understanding of the molecular process involved will be essential if the clinical potential of these cells is to be realized. The recent observations of stem-cell plasticity suggest that perhaps our current concepts about the operation of cell regulatory pathways are inadequate, and that new approaches for analysing complex regulatory networks will be essential.

Keywords: embryonic stem cells; teratocarcinoma; embryonal carcinoma; human; differentiation; plasticity

1. INTRODUCTION

The present flurry of interest in stem cells and their potential for 'regenerative' medicine has arisen from the confluence of several streams of biological thought and investigation. The first is the notion of stem cells themselves, which evolved among biologists considering the regeneration of tissues that are necessarily replaced throughout adult life, most obviously the blood, the lining of the gut and the skin. It was proposed that the replacement of these complex tissues, comprising many different cell types, depends upon the existence of small populations of undifferentiated cells, 'stem cells', that not only proliferate to replace themselves indefinitely ('self-renewal'), but also differentiate into daughter cells with a limited proliferative capacity. These so-called 'transit amplifying' cells are committed to differentiate further to a restricted range of terminal cell types responsible for the principal functions of such tissues (Lajtha 1979; Potten & Lajtha 1982). Such terminally differentiated cells typically lose their capacity for further proliferation.

This type of tissue architecture may very well have a selective advantage in minimizing the susceptibility of an organism to cancer, which is almost certainly the result of the accumulation of mutations that affect both cell proliferation and differentiation. It was pointed out by Pierce (1974) that many cancers seem to be caricatures of their tissue of origin, with a malignant stem cell similar to the stem cell of the healthy tissue, as well as a variety of cells at progressive stages of differentiation. He proposed that malignancy is a consequence of a failure to regulate the balance between proliferation and differentiation, resulting in the accumulation of cells at earlier stages of the differentiation pathways. Where the issue has been analysed in detail, notably in the haematological malignancies, possibly only few cells within a tumour mass, the so-called 'clonogenic cells', are capable of transferring a malignancy to a new host (Greaves 1982). Cairns (1975) pointed out that it is mutations occurring in the stem-cell population that are most likely to become 'fixed' in a tissue and cause long-term damage because mutations in transit amplifying and terminally differentiated cells would normally be lost as the tissue turned over. He suggested that there would then be a selective advantage for an organism to maintain only small stem populations to minimize the target for mutagenesis, and hence carcinogenesis.

Many tissues that generally appear quiescent also turn over slowly (e.g. bone), or may proliferate or regenerate in response to physiological cues (mammary tissue in pregnancy) or injury (liver). Presumably these, too, retain stem cells, or cells that are capable of conversion into stem cells. But it has now become evident that tissues not previously thought capable of regeneration, notably the nervous system, also harbour stem-cell populations (Temple 2001). However, the results that have caused general surprise come from a series of experiments, by different groups working on different organ systems, suggesting that stem cells from one tissue, hitherto thought committed to the lineages of that tissue, can give rise to distinct cell types of embryologically unrelated tissues under some circumstances. Thus, evidence has been presented for neural stem cells giving rise to blood cells (Bjornson *et al.* 1999) and to muscle (Galli *et al.* 2000), haematopoietic stem cells giving rise to neurons (Brazelton *et al.* 2000; Mezey *et al.* 2000), muscle (Goodell *et al.* 2001) and liver (Alison

et al. 2000; Peterson *et al.* 1999; Lagasse *et al.* 2000), muscle stem cells giving rise to blood cells (Jackson *et al.* 1999; Seale *et al.* 2001), and mesenchymal stem cells from bone giving rise to a wide variety of tissues (Pittenger *et al.* 1999). Perhaps the most striking observation was that neural stem cells transferred to a blastocyst could contribute to tissues belonging to all three germ layers (Clarke *et al.* 2000).

Many questions still remain about these observations of apparent stem-cell plasticity; only in a few cases have the experiments been conducted with clonal, well-characterized stem cells—a necessity if one is to conclude that a particular stem cell is able to give rise to several distinct cell types. In any case, if we take the results at face value, are we observing a normal physiological function of stem cells, or are we seeing the consequences of rare switches in their differentiative capacity or potency? In the latter case, is this because of the exposure of the cells to environments that they do not normally see—an issue of the difference between what cells 'can do'—'prospective potency'—and what they normally do when undisturbed—'prospective fate' (Weiss 1939). Or are the changes in their patterns of differentiation due to rare events involving intrinsic changes to the patterns of gene activity that regulate the determined state of a cell? Regardless of the answers to these questions, the observations have nevertheless raised the prospect of clinically using stem cells from adult tissues in cell replacement therapies designed to repair or replace tissues damaged by accident or disease.

The second stream of work contributing to the current debate is one that began with a long-standing fascination with a peculiar type of tumour, the teratomas, and has culminated in the derivation of stable, pluripotent human embryonic stem (ES) cell lines in culture. Teratomas present a haphazard array of cell differentiation that appears to recapitulate many of the events that occur during early embryonic development but in a disorganized manner (Damjanov 1993; Dixon & Moore 1952; Mostofi & Price 1973). They have intrigued clinicians and biologists for many hundreds of years; excellent summaries of the history of this interest are provided by Damjanov & Solter (1974) and Wheeler (1983). The experimental study of teratomas began with the discovery by Stevens & Little (1954) that they occur spontaneously in the testes of about 1% of male mice of the 129 strain, or that they can be induced by transplanting the genital ridges of embryos from strain 129 mice, and a few other strains, to the testes of adult mice (Stevens 1964, 1970*a*,*b*). Some of these tumours were evidently malignant and could be re-transplanted to successive hosts. Such 'teratocarcinomas', as the re-transplantable, malignant tumours were known, contained a relatively undifferentiated cell type known as an 'embryonal carcinoma' (EC) cell, long suspected as the stem cell of the tumour. This stem-cell character of EC cells was confirmed by Kleinsmith & Pierce (1964), who showed that single EC cells transferred to a new host could reform a complex teratocarcinoma that could again be re-transplanted to another host. A long series of experiments through the 1960s and 1970s, seeking to characterize these EC cells and their relationship to embryonic cells, culminated with the isolation, in 1981, of pluripotent cells from very early mouse embryos (Evans & Kaufman 1981; Martin 1981) and eventually, in 1998, from human embryos (Thomson *et al.* 1998). These 'normal' embryo-derived cells became known as ES cells. In fact, the term 'stem cell' was rarely used previously by embryologists (e.g. Wilson 1896) and it then generally referred to precursor cells within the developing embryo. Such cells usually exist only transiently, and their self-renewal is normally limited, unlike 'stem cells' in the adult. Although ES cell lines in culture do meet the criteria used in the context of 'adult stem cells' of indefinite self-renewal together with a capacity for differentiation, the embryonic cells of the late inner cell mass (ICM), to which they correspond, generally disappear from the developing embryo by the time of gastrulation. However, it is intriguing to speculate that a number of childhood cancers may arise because of the abnormal persistence of 'stem cells' present during embryogenesis.

The third stream of relevant research encompasses the work of those who sought first to determine whether terminal differentiation of cells during embryogenesis results from the loss of genes, or whether altered regulation without a significant change in the complement of genes present in a cell could be responsible. Following earlier studies of Briggs & King (1952), Gurdon (1962) showed that, in *Xenopus laevis*, nuclear transfer from differentiated somatic cells to enucleated oocytes supported embryonic development, indicating that the nuclei of differentiated cells in amphibians contain a full genetic complement. These experiments incidentally demonstrated an approach for producing 'clones' of genetically identical individuals. For many years it seemed that this might not be possible in the case in mammals (McGrath & Solter 1984), and definitive evidence that mammalian 'cloning' by nuclear transfer from somatic cells is possible was not acquired until experiments with sheep embryos led to the birth of 'Dolly', born following the transfer of a nucleus from a mammary cell of an adult sheep to an enucleated oocyte (Campbell *et al.* 1996; Wilmut *et al.* 1997). That observation was quickly followed by finding that 'cloning' is also possible in mice (Wakayama *et al.* 1998), cows (Cibelli *et al.* 1998) and pigs (Onishi *et al.* 2000). It is a short step to imagine that this would also be possible in humans, if anyone chose to take this step.

The idea of using such nuclear transfer techniques for so-called 'reproductive cloning' in humans is an anathema to most scientists, not least because the process is both inefficient and subject to serious errors so that most animals developing by this route have proved to be defective. However, it rapidly occurred to several people that embryo cloning by this route, coupled with the ability to derive ES cells from very early embryos at the blastocyst stage, could allow the development of almost any differentiated cell type that would be genetically identical to a prospective patient, who could then receive such cells in tissue replacement therapies (essentially autografts), the so-called 'therapeutic cloning' approach. As a proof of concept, Munsie *et al.* (2000) have isolated murine ES cells from blastocysts derived by somatic nuclear transplantation. The derivation of human embryos following somatic nuclear transfer to an enucleated oocyte has also been reported, though subsequent development arrested prior to blastocyst formation at the six-cell stage (Cibelli *et al.* 2001). So far, no one has described the derivation

of human ES cells from such embryos. Whether it will ever prove a practicable proposition to derive cells tailored for specific patients, instead of finding other ways for defeating the immune system to permit allografts from established, non-autologous ES lines, is a moot point.

Thus, the current excitement about the potential of stem-cell biology for regenerative medicine arises in part from the identification and culture of various types of stem cells, whether from embryos or adults, and in part from results indicating that adult stem-cell plasticity or embryo cloning by somatic cell nuclear transplantation may be realistic approaches for providing autologous tissues for grafting to evade problems of immunological incompatibility. Nevertheless, it is arguable that, despite considerable progress in the biology of stem cells from various adult tissues, ES cells from the early embryo are perhaps the best understood. Clear identification of the various adult stem cells has often been elusive; some, notably haematopoietic stem cells, cannot be easily cultured and expanded *in vitro*, while others require continual re-isolation as indefinite culture *in vitro* does not seem possible. By contrast, ES cells from mice and humans have been cloned (Amit *et al.* 2000), in the sense that it has been shown that single identifiable cells can give rise to a variety of distinct cell types by differentiation, they can be cultured and expanded *in vitro*, apparently indefinitely, without loss of potency, and they could, in principle, be used to generate all tissues of the body. These features mean that it should be possible establish an inexhaustible supply of well-characterized cells for clinical use—an important issue for ensuring safe protocols. It is also important to recognize that ES cells, and their malignant EC cell counterparts, provide invaluable tools for analysing cell differentiation throughout embryogenesis, which also has implications for understanding diseases such as cancer. In turn, ES cells may also provide keys for understanding the biology of 'adult stem cells'.

2. THE BIOLOGY OF TERATOCARCINOMAS

Teratomas are generally benign tumours that occur most commonly in the ovary, where they are also known as benign ovarian cysts (figure 1*a*). These arise from oocytes that have undergone parthenogenetic activation, begun development and then become disorganized to form a mass of embryonic tissue (Stevens & Varnum 1974). Teratomas are also found, though more rarely, in other sites, including the base of the spine in newborn infants.

Similar tumours occur in the testis, but in this case they are generally highly malignant and consequently known as teratocarcinomas (Dixon & Moore 1952; Mostofi & Price 1973). These form a subgroup of the germ-cell tumours (GCTs) that account for almost all testicular cancers. Testicular GCTs, which appear to arise from abnormal gonocytes within the seminiferous tubules (figure 1*b*) (Skakkebaek 1972) are rare, but have a peak incidence in young post-pubertal men making them the most common malignancy in this age group (Møller 1993). Furthermore, their incidence has increased dramatically over the past 50 years. The uncommonly young age of GCT patients contributes to their medical significance but, fortunately, GCT are amongst the most treatable cancers since the advent of *cis*-platinum-based therapy in the 1970s (Einhorn 1987; Stoter 1987).

GCTs are typically divided into seminomas and non-seminomas (Damjanov 1990, 1993). Seminomas consist of cells that resemble primordial germ cells; they do not occur in mice. In contrast, non-seminomas are histologically heterogeneous and frequently contain somatic tissues such as nerve, bone, muscle, etc.; sometimes they contain structures, embryoid bodies, in which these cells are organized to resemble closely an early embryo (figure 1*c*,*d*). These tumours also contain histologically undifferentiated elements composed of EC cells, the key malignant pluripotent stem cell of these tumours. The term teratocarcinoma is generally used for tumours containing both EC and teratoma components.

In 1954, Stevens & Little reported that males of the 129 strain develop spontaneous testicular teratomas and teratocarcinomas that can be observed as incipient tumours, forming structures described as embryoid bodies within the seminiferous tubules of the developing gonad, as early as 13 days of embryonic development (Stevens 1964). Stevens also showed that these tumours can be induced experimentally in strain 129 mice and a limited range of other strains by explanting genital ridges of foetuses, between 11 and 13.5 days of development, to ectopic sites (Stevens & Hummel 1957; Stevens 1967*a*, 1970*a*). These results suggested that the origins of teratomas were from primordial germ cells as, in the mouse embryo, these migrate into the genital ridge at 11 days (Bendel-Stenzel *et al.* 1998). The upper limit of 13 days implies some further changes in the germ cells, which could be associated with their entering mitotic arrest soon after their arrival in the genital ridge. Confirmation of the germ-cell origin of the spontaneous and experimental testicular teratomas came from studies of mice homozygous for the *Steel* (*Sl*) mutation (Stevens 1967*b*). Viable *Sl*/*Sl* homozygotes are infertile as the primordial germ cells do not survive migration. Stevens found that the genital ridges from homozygous *Sl*/*Sl* 129 mice did not yield teratomas.

Although, testicular teratomas can only be induced in a limited number of strains, teratomas can also be formed from many strains of mice by the transplantation of rather earlier embryos, at the egg cylinder stage (about 7 days of development) to ectopic sites (Solter *et al.* 1970, 1979, 1981). As in the spontaneous ovarian tumours derived from parthenogenotes, these embryos become disorganized and form teratomas or teratocarcinomas, depending upon the host strain into which the embryo is transplanted and, interestingly, not upon the genotype of the embryo itself. Curiously, the range of mouse strains from which teratomas and teratocarcinomas can be derived by this route, and so from which EC cell lines can be established, is considerably greater than the range of strains from which ES cells have been derived by blastocyst culture, or teratocarcinomas from genital ridges, whether spontaneously or by transplantation. These results raise the question of what is the cell of origin of the EC cells derived from egg cylinder explants—presumably they are neither ICM/epiblast cells nor primordial germ cells. A further curiosity is that, apart from the spontaneous human tumours, it has not proved possible to derive teratocarcinomas routinely by such techniques in other species, though in the rat the yolk sac can give rise

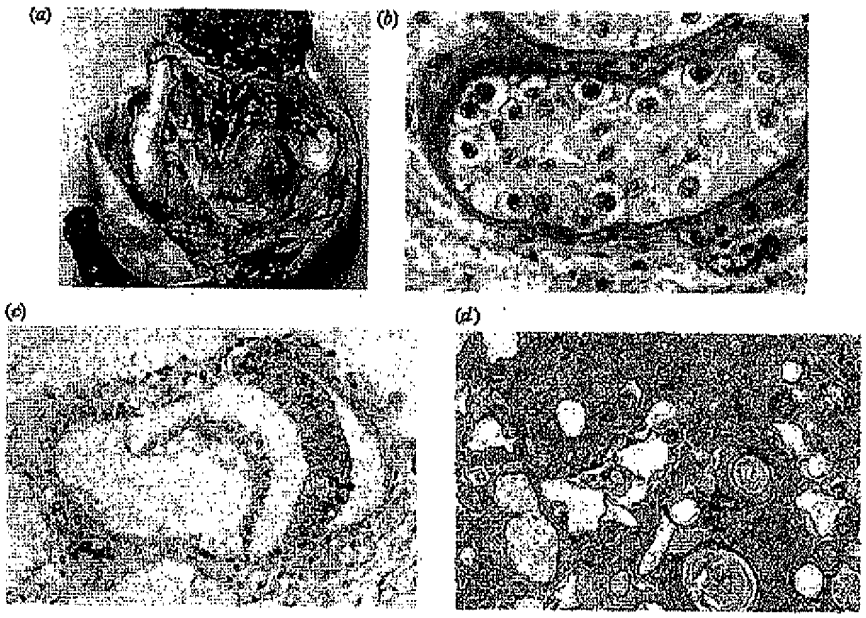

Figure 1. Examples of ovarian and testicular teratocarcinomas. (*a*) A mature human teratoma, or dermoid cyst of the ovary, showing hair and a tooth. (*b*) Carcinoma *in situ*: the pre-invasive phase of human testicular teratocarcinomas in which abnormal gonocytes fill the seminiferous tubules of the testis. (*c*) An embryoid body within a human testicular teratocarcinoma. (*d*) A well-differentiated mouse teratocarcinoma. Note extensive differentiation including keratin pearls and cartilage.

to a teratoma after the fetus is surgically removed (Sobis *et al.* 1993). These rat tumours are not re-transplantable and the isolation of a rat EC, or indeed ES, cell line has proved elusive.

3. EMBRYONAL CARCINOMA CELL LINES

Further study of the biology of mouse EC cells awaited the derivation of EC cell lines established in culture, which was first reported by Finch & Ephrussi (1967) and Kahn & Ephrussi (1970). Subsequently, several groups established murine EC cell lines throughout the 1970s (Bernstine *et al.* 1973; Jakob *et al.* 1973; Evans 1972; Martin & Evans 1974, 1975; Nicolas *et al.* 1975). Many of these lines remained pluripotent and, although they could be maintained as undifferentiated EC cells under certain culture conditions, they retained the ability to form teratocarcinomas when transplanted back into an appropriate mouse host. Many of the pluripotent lines would also differentiate in culture, but the circumstances varied between lines: some EC cell lines remained undifferentiated if kept proliferating in subconfluent cultures, but differentiated spontaneously to yield a variety of cell types, including nerve and muscle, if maintained in confluent cultures for several days (Nicolas *et al.* 1975). Other pluripotent EC lines required culture on feeder layers of transformed mouse fibroblasts to prevent differentiation (Martin & Evans 1974, 1975). In this case, differentiation could be induced by removing the EC cells from the feeder cells, and particularly if the cells were grown in suspension when they aggregated to form structures known as embryoid bodies with an inner core of EC cells surrounded by a layer of cells resembling the visceral endoderm of an early mouse conceptus. Gradually these embryoid bodies became histologically complex and a wide variety of differentiated cells would grow out when they plated on a substrate that permitted attachment.

The uncontrolled nature of spontaneous differentiation of EC cells, although dramatic, made study of the underlying processes difficult. The discovery by Strickland & Mahadavi (1978), that an apparently nullipotent EC cell line, F9, could be induced to differentiate by exposure to retinoic acid, proved a significant advance. After exposure to both retinoic acid and cAMP these F9 EC cells generated cells that closely resemble parietal endoderm (Strickland *et al.* 1980). Subsequently, it was found that if F9 cells are allowed to form clusters in suspension in the presence of retinoic acid, they form embryoid bodies in which the outer layer of cells resembles visceral endoderm, while the inner cells retained an EC phenotype (Hogan *et al.* 1983). Thus, apparently F9 cells can be switched between differentiating into visceral or parietal endoderm depending upon external cues. It was later shown that retinoic acid, as well as other agents, notably dimethylsulphoxide (DMSO) and hexamethylene bisacetamide (HMBA), could also induce the differentiation of a number of mouse EC cell lines (Jakob *et al.* 1978; McBurney *et al.* 1982). In particular, at least in the case of the P19 EC cell line, retinoic acid tended to induce neurectodermal differentiation, whereas DMSO induced mesoderm differentiation and the appearance of cardiac muscle (Jones-Villeneuve *et al.* 1982).

4. RELATIONSHIP TO THE EARLY EMBRYO

During the 1970s, it was found that murine EC cells express various features that are typical of cells of the ICM and primitive ectoderm of the early conceptus (Artzt *et al.* 1973; Jacob 1978). Following the line of reasoning that EC cells are a malignant equivalent of those embryonic cell types, EC cells from tumours were transplanted into blastocysts that were then re-implanted into the uterus of pseudo-pregnant female mice (Brinster 1974; Papaioannou *et al.* 1975; Mintz & Illmensee 1975). Under these conditions, the EC cells, which would typically form teratomas if transplanted to ectopic sites of an adult mouse, often appear to become normalized under the influence of the blastocyst and to participate in embryonic development giving rise to a range of normal tissues in the chimeric mice that subsequently develop.

In rare cases, it was reported that the germ cells of such chimeric mice also derived from the transplanted EC cells, but these observations have not been confirmed. Despite early indications to the contrary, it appears that many EC cells when transplanted into blastocysts do give rise to teratomas in the developing mice, so that their tumour characteristics are not completely suppressed by the host embryo. The latter result is, perhaps, not surprising as EC cells have generally adapted over many generations to growth either in culture or as a tumour in a host mouse. Because their differentiated derivatives usually have a limited lifespan and are non-malignant, one can easily imagine that the accumulation of mutations that inhibit differentiation would provide the EC cells with a selective advantage. When mouse EC cells are fused with somatic cells, notably thymocytes from adult mice, the resulting hybrid cells often exhibit the general features of EC cells (Miller & Ruddle 1976; McBurney 1977; Rousset *et al.* 1980; Gmür *et al.* 1980). However, in several cases, it has been noted that these hybrid EC cells show a greater capacity for differentiation than their EC parents (Andrews & Goodfellow 1980; Rousset *et al.* 1983). The simplest explanation for these observations is that the normal genome of the somatic cell introduces genes that are capable of complementing accumulated EC cell mutations that have tended to inhibit their ability to differentiate.

The recognition that EC cells are the malignant counterparts of embryonic ICM cells eventually resulted in the experiments of Evans & Kaufman (1981) and Martin (1981), who showed that it is possible to derive permanent lines of cells directly from mouse blastocysts, which closely resemble the EC cells derived from teratomas. They termed these cells ES cells. The normal cells to which these lines are thought to be equivalent, namely the cells of the late ICM, do not normally persist for any great length of time. The apparent ability of ES cells to grow indefinitely and exhibit an immortal characteristic, i.e. to present classical 'stem-cell features', seems to be a consequence of their removal from the embryo and maintenance in tissue culture. A further development, stimulated by the origins of testicular teratocarcinomas from primordial germ cells, was the finding that when such primordial germ cells are cultured *in vitro* they convert to cells, called embryonic germ (EG) cells, that closely resemble EC and ES cells (Matsui *et al.* 1992; Resnick *et al.* 1992).

5. HUMAN EC AND ES CELL LINES

Human teratocarcinoma cell lines were first isolated in the 1950s as xenografts in hamster cheek pouches (Pierce *et al.* 1957). Several lines were subsequently established *in vitro* during the 1970s, notably TERA1, TERA2 (Fogh & Trempe 1975) and SuSa (Hogan *et al.* 1977). Many of these human lines showed little capacity for differentiation, but they provided the basis for the identification of a number of characteristic features of human EC cells. Eventually, several human EC cell lines capable of differentiation were obtained, including: GCT27 (Pera *et al.* 1989; Roach *et al.* 1993, 1994; Pera & Herszfeld 1998), NCCIT (Teshima *et al.* 1988; Damjanov *et al.* 1993) and NCG.R3 (Hata *et al.* 1989; Umezawa *et al.* 1996) and, ironically, TERA2 (Andrews *et al.* 1984*b*), one of the oldest extant human teratocarcinoma cell lines. The pluripotent character of TERA2, and indeed its identity as an EC cell line, was overlooked for some time (e.g. see Andrews *et al.* 1980) because the specific culture conditions required to maintain pluripotent, undifferentiated human EC cells were not fully appreciated. Thus, in contrast to many mouse EC lines, it is necessary to maintain human EC cells at high cell densities, and it is generally best to passage TERA2 by scraping rather than by using trypsin to harvest the cells—retaining the cells in small clumps, which would be disrupted by trypsinization, seems to inhibit spontaneous differentiation. The widely used clonal subline of TERA2, NTERA2 cl.D1 (NT2/D1), was re-isolated after passage of a well-differentiated culture of TERA2 as a xenograft tumour in a nude mouse, which appeared to 'rescue' persisting EC cells within the culture (Andrews *et al.* 1984*b*).

Human and mouse EC cells differ significantly from one another, although they share some common features. For example, their morphology and growth patterns are similar, as both tend to grow in clusters of tightly packed cells with relatively little cytoplasm and prominent nucleoli. Also, both express high levels of alkaline phosphatase (Bernstine *et al.* 1973; Benham *et al.* 1981). However, the differences include a distinct pattern of surface antigen expression, as well as a propensity for human but not murine EC cells to differentiate into trophectoderm (Andrews *et al.* 1980, 1982, 1984*b*, 1996; Damjanov & Andrews 1983). The specific features of murine EC cells that differ from human EC cells, notably their expression of the lactoseries glycolipid antigen, stage-specific embryonic antigen 1 (SSEA1) (Kannagi *et al.* 1982; Gooi *et al.* 1981; Solter & Knowles 1978), their lack of expression of two globoseries glycolipid antigens, SSEA3 (Shevinsky *et al.* 1982) and SSEA4 (Kannagi *et al.* 1983), and their lack of ability to differentiate into trophectoderm, are all shared with murine ES cells, consistent with the idea that they resemble embryonic cells of the ICM and primitive ectoderm: although SSEA3 and SSEA4 are expressed by cleavage stage mouse embryos, the ICM lacks these antigens, but expresses SSEA1. Further, the ICM cells soon lose the capacity for trophectodermal differentiation. Thus, without the existence of human ES lines and without direct information from human embryos, the differences between human and mouse EC cells made the relationship of human EC cells to the early human embryo uncertain.

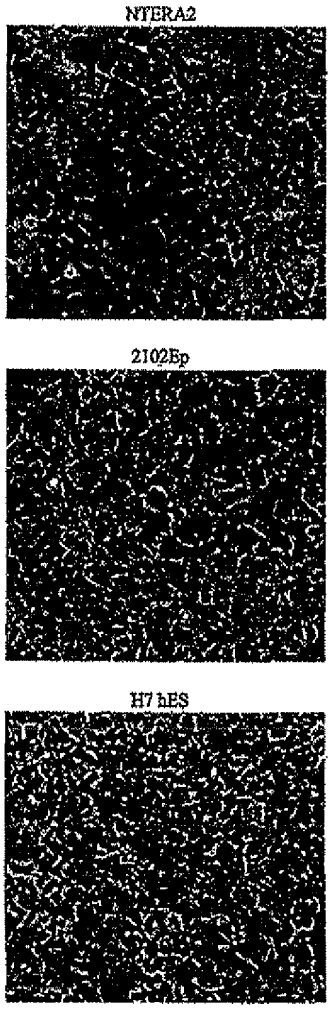

Figure 2. A comparison of the morphology of a human ES cell line, H7, provided by Dr James Thomson, and two human EC cell lines: NTERA2, capable of extensive differentiation (Andrews *et al.* 1984*b*), and 2102Ep, capable of only limited differentiation (Andrews *et al.* 1982). Note the tightly packed cells with little cytoplasm, pale nuclei and prominent nucleoli.

Logistical problems, as well as legal and ethical ones, delayed the derivation of ES cell lines from human embryos. However, rhesus monkey ES cell lines were first reported in 1995 (Thomson *et al.* 1995) and marmoset ES lines in 1996 (Thomson *et al.* 1996). These primate ES cells more closely resemble human EC cells than murine EC and ES cells. Eventually the derivation of human ES cell lines was reported by Thomson *et al.* (1998) and Reubinoff *et al.* (2000). These human ES cells also resemble the rhesus monkey ES cells and human EC cells (figure 2) rather than the murine cells—they are able to differentiate into trophoblast and are SSEA3(+), SSEA4(+), SSEA1(−). Also like human EC cells, but not those of the mouse, they express class I MHC antigens and Thy1 (Draper *et al.* 2002). Conversely, human ES cells do resemble the mouse cells in their expression of alkaline phosphatase and the stem-cell-specific transcription factor, *Oct4* (Yeom *et al.* 1996). Recently, we have confirmed that indeed the ICM of human blastocysts resembles human EC and ES cells in several of these features (Henderson *et al.* 2002), notably the expression of SSEA3 and SSEA4, the lack of SSEA1 and also the expression of two human cell-surface, keratan sulphate-related antigens, TRA-1-60 and TRA-1-81, which were previously defined on human EC cells (Andrews *et al.* 1984*a*; Badcock *et al.* 1999). Thus, there do appear to be significant differences in the biology of early embryonic cells of human and mouse embryos.

The precise significance of these differences remains to be resolved, especially as the function of the surface antigen markers, mostly involving carbohydrate epitopes, is unknown. Nevertheless, the regulation of these molecules is subject to tight control during differentiation; SSEA1 has been suggested to mediate compaction in mouse embryos (Bird & Kimber 1984; Fenderson *et al.* 1984), and a role for SSEA3 and SSEA4 in embryo pathology has been suggested (Tippet *et al.* 1986). SSEA3 and SSEA4 belong to the P-blood-group system and are expressed on red cells, except in individuals with the pp or p^k phenotypes. Women with these latter phenotypes are reported to be susceptible to recurrent early spontaneous abortions (Race & Sanger 1975); the expression of SSEA3 and SSEA4 by early embryonic cells suggests that these could be the target for a proposed immune response to the early embryos in such women. However, regardless of the functional significance of these particular differences between mouse and human embryos, the results show that lessons from the mouse cannot necessarily be extrapolated to human embryogenesis. They also suggest that human EC cells remain a useful complement of human ES cells for exploring the mechanisms that regulate human development; while ES cells are 'genetically normal' and differentiate into a wide range of cell types, EC cells are easier to grow and exhibit a simplified pattern that can be analysed more easily.

Human EG cells have also been described but, although pluripotent, they seem to differ from human EC and ES cells with respect to various surface antigen markers (Shamblott *et al.* 1998). However, the significance of these differences remains to be resolved.

6. DIFFERENTIATION OF HUMAN EC AND ES CELLS

Although EC cells are the stem cells of teratocarcinomas, a striking feature of many human EC cell lines is their lack of ability to differentiate into well-recognizable cell types. This might, in part, reflect their origins in tumours, as the acquisition of an inability to differentiate could provide a strong selective advantage. Human EC cells are highly aneuploid and it is easy to envisage that genetic changes might occur to inhibit their differentiation. Indeed, cell-hybrid studies with both mouse and human EC cells support this idea and suggest that a loss of pluripotency results from the loss of key gene functions

(Andrews & Goodfellow 1980; Duran *et al.* 2001). Nevertheless, several human EC cell lines can differentiate well and have been studied extensively. One line, GCT27, requires maintenance on feeder layers and differentiates into a wide range of cell types when removed from the feeders (Pera *et al.* 1989). Another human EC cell line, TERA2 and its NTERA2 sublines, does not require feeders and differentiates extensively, but in distinct directions, in response to retinoic acid (Andrews 1984), HMBA (Andrews *et al.* 1990) and the bone morphogenetic proteins (BMPs) (Andrews *et al.* 1994).

Apart from forming well-differentiated teratomas when grown as xenografts in nude mice, TERA2 and NTERA2 cells rapidly lose their EC phenotype and differentiate into a wide array of cell types, including neurons, after exposure to 10^{-5} or 10^{-6} M retinoic acid (Andrews 1984). This differentiation is marked by a switch in glycolipid synthesis from globoseries to lactoseries and ganglioseries structures (Fenderson *et al.* 1987), and can conveniently be followed by the loss of antigens, such as SSEA3 and SSEA4, as well as TRA-1-60 and TRA-1-81, and the acquisition of antigens, such as SSEA1, A2B5 (ganglioside GT3) and ME311 (ganglioside 9-*O*-acetyl GD3). There are also substantial changes in gene activity, most notably activation of the *HOX* genes in a retinoic acid concentration-dependent manner (Simeone *et al.* 1990), and the appearance of susceptibility to infection and replication of human cytomegalovirus (Gönczöl *et al.* 1984) and human immunodeficiency virus (Hirka *et al.* 1991), neither of which will grow in the undifferentiated EC cells.

As differentiation progresses, neural markers become evident, and neurons that express neurofilament proteins and a typically neuronal morphology appear (figure 3*a*) after one to two weeks (Andrews 1984; Lee & Andrews 1986). The pattern of expression of genes related to the early stages of neural differentiation during embryonic development—nestin in mitotic neural precursors and NeuroD in post-mitotic neuroblasts—seems to be followed by differentiating NTERA2 cells (Przyborski *et al.* 2000; Pleasure *et al.* 1992; Pleasure & Lee 1993). Furthermore, the terminal neurons are functional and express tetrodotoxin-sensitive sodium channels (Rendt *et al.* 1989), glutamate receptors and voltage-gated calcium channels (Squires *et al.* 1996). Several studies have recently been conducted of their potential for implanting into the central nervous system, initially in experimental rats in which NTERA2-derived neurons will apparently integrate functionally to correct neural defects, such as those resulting from stroke (Borlongan *et al.* 1998; Hurlebert *et al.* 1999; Kleppner *et al.* 1995; Philips *et al.* 1999). Experiments to implant these EC-cell-derived neurons into human stroke patients have also been reported (Kondziolka *et al.* 2000). The tumour origins of these neurons provokes some disquiet about safety, and whether their implantation will provide benefits to patients remains to be seen. Nevertheless, the experiments pave the way for future studies using neurons derived from genetically normal ES cells.

When differentiation of NTERA2 EC cells is induced by HMBA or BMP7, distinct differences from retinoic-acid-induced differentiation are seen (Andrews *et al.* 1990, 1994). Thus, ganglioside antigens are only expressed late, on a small proportion of cells, and few neurons are evident. The identity of many of the cells induced by HMBA and BMP has not been clearly defined, although smooth muscle actin was particularly noted after BMP induction. One idea we have considered is that NTERA2 differentiates predominantly in an ectodermal direction but that, depending upon the conditions, this can take a more 'dorsal' (predominantly neural) or 'ventral' (epidermal) character. By this notion, retinoic acid may favour a more dorsal pattern of differentiation, whereas HMBA and BMP may promote a more ventral pattern.

Not surprisingly, the embryo-derived human ES cells exhibit a considerably greater potential for differentiation. When grown in immunodeficient mice, these cells form well-differentiated teratomas with well-organized tissues representing all three germ layers (Thomson *et al.* 1998). When grown *in vitro*, as for mouse ES cells, the human ES cells require maintenance on feeder layers to prevent differentiation, but the cytokine LIF, which is able to prevent the differentiation of mouse ES cells in the absence of feeders, is apparently not able to do so for human ES cells. Conversely, it has been reported that conditioned medium from fibroblasts will inhibit differentiation of cells grown on Matrigel (Xu *et al.* 2001).

Many studies of differentiation in human ES cell cultures have focused upon allowing the cells first to form 'embryoid bodies' by culture in suspension; after a period of suspension culture, a variety of differentiated cells can be detected in the resulting cell clusters, which will grow out when allowed to attach. Under these conditions, neurons, glia, skeletal and cardiac muscle, liver and even insulin-secreting islet cells have been reported (Assady *et al.* 2001; Kaufman *et al.* 2001; Kehat *et al.* 2001; Itskovitz-Eldor *et al.* 2000; Odorico *et al.* 2001; Pera 2001; Schuldiner *et al.* 2000).

Differentiation also occurs in attached cultures, either spontaneously, or by induction with agents such as retinoic acid. Even when maintained on feeders, colonies of clearly non-ES cells can often be seen, and gene expression indicative of extra-embryonic cell types (e.g. chorionic-gonadotropin-indicating trophoblast and α-fetoprotein-indicating yolk sac) can be detected in the SSEA3(−) presumptive non-ES cells isolated by 'fluorescence-activated cell sorting' from such cultures (Henderson *et al.* 2002). These spontaneous differentiated derivatives can present a problem to continued maintenance of the ES lines if they are allowed proliferate, and culture techniques need to be adapted to remove them during passaging.

Retinoic acid, HMBA and DMSO also induce differentiation of attached cultures, whether in the presence or absence of feeders (Draper *et al.* 2002). Many of the changes seen during the differentiation of human EC cells induced by these agents are also seen in the human ES cultures. For example, all the typical EC/ES marker antigens, SSEA3, SSEA4, TRA-1-60, TRA-1-81, etc. are downregulated. Also, neural differentiation is common (figure 3*b*). However, although the induction of some of the antigens that appear during EC differentiation is seen, there are many differences in the patterns of antigens observed, probably because the ES cells are capable of a much wider range of differentiation.

Although it is clear that human ES cells are capable of extensive differentiation, as in the mouse, and although

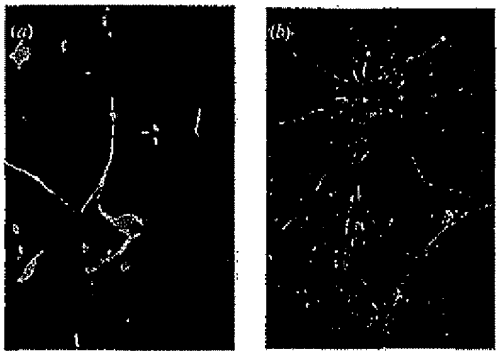

Figure 3. (*a*) Neurons differentiating from NTERA2 human EC cells induced with retinoic acid, and (*b*) a low-power view of an extended network of neurons in a culture of human ES cells (H7), also induced with retinoic acid. Both are stained with an antibody to the 200 kDa neurofilament protein.

various protocols are being developed to favour particular lineages of differentiation, we currently know very little of the mechanisms that regulate their decision to differentiate in the first place, or that regulate which lineages are followed once differentiation is initiated. Answers to these fundamental questions could provide valuable insights into a wide range of issues pertinent to human health, from the causes of abnormal embryonic development and the formation of birth defects, to understanding the relationships between cell proliferation and differentiation that underlie the development of cancer. Such answers are also crucial if ES cells are to realize their promise of providing tissues for transplantation and 'regenerative' medicine. Thus, efficient maintenance of ES cells suitable for use in human patients will demand culture conditions that minimize not only spontaneous, unwanted differentiation, but also the use of animal products, whether feeder cells, serum or growth supplements, which could introduce adventitious pathogens. Equally, although a wide range of differentiation is easy to obtain, particular cell types often only constitute a small proportion of a differentiated culture. Efficiency of production, apart from the need to avoid contamination with unwanted cell types that may be deleterious, demands that culture techniques are developed to promote differentiation in only specific directions.

Although murine EC and ES cells were originally derived with approaches to addressing such questions in mind, most of the use of mouse ES cell technology over the past 20 years has been directed towards production of transgenic mice, and not for answering questions of fundamental cell biology pertinent to ES cells *per se*. Nevertheless, some studies of the mechanisms that regulate mouse ES cell differentiation have been carried out. Most notable are studies that have highlighted the crucial role of specific discrete levels of *Oct4* expression for the maintenance of an undifferentiated phenotype (Niwa *et al.* 2000). Thus, if the level of *Oct4* expression in mouse ES cells is reduced, differentiation into trophectoderm ensues, whereas if levels are raised, differentiation to extra-embryonic endoderm is promoted. Furthermore, the maintenance of an undifferentiated phenotype depends upon activation of the STAT3 signalling pathway and inhibition of the MAPK/ERK pathway, following interaction of LIF with its receptor (Niwa *et al.* 1998; Burdon *et al.* 1999; Boeuf *et al.* 1997). However, as LIF does not appear to influence the behaviour of human ES cells (Thomson *et al.* 1998; Reubinoff *et al.* 2000), it remains uncertain to what extent these lessons will apply to human ES cells. It is possible that the STAT3 and MAPK pathways could be regulated by other receptor ligand systems; alternatively, other signalling pathways, yet to be identified, might perform similar functions in humans.

7. CONCLUDING THOUGHTS

A common notion that has prevailed in developmental biology for many years is one of cell differentiation during embryogenesis proceeding through a series of successive binary decisions by which cells adopt alternative phenotypes. Thus, embryogenesis is commonly seen in terms of cells following branching pathways of differentiation. Waddington (1956, 1966) has described such a process as 'canalization', envisaging cells moving 'downhill' through a series of valleys making up the 'epigenetic landscape' (figure 4*a*). A further notion that is often linked to this concept, although by no means essential, is that the process of differentiation is unidirectional and, in normal circumstances, irreversible. The branching pathways are seen as representing successive commitment of cells progressively to restricted options of eventual cell fate.

While the idea of 'de-differentiation', implying reversal of specific steps of differentiation, has often been discussed, particularly in the context of tumour biology, it has not found general favour. In the case of cancers that appear, for example, to express so-called 'oncofetal' proteins, it may be more appropriate to consider that it is the stem cells of specific tissues that are the target for carcinogenesis, and that it is their overgrowth that gives the appearance of reversion, or 'de-differentiation' (Pierce 1974). However, the recent reports of stem cells from different adult tissue, displaying quite unexpected plasticity and apparent lack of specific commitment, suggests that perhaps the concepts of unidirectional, irreversible differentiation along distinct cell lineages should be revised. Indeed, 'trans-differentiation', as in metaplasia, is well known to pathologists, while 'trans-determination' of imaginal disc cells in *Drosophila* was described many years ago by Hadorn (1968).

When considering the factors that regulate cell behaviour, whether commitment and determination, or differentiation, attention commonly focuses on individual signalling pathways by which cells respond to external cues, e.g. growth factors, the extracellular matrix, or interactions with other cells. To keep the analysis simple, such signalling pathways within a cell are often considered in isolation, and are also considered as simple switches—either 'on' or 'off'. However, any molecules within a signalling pathway will obey the normal chemical laws affecting reaction rates and equilibria. The activity of particular regulatory molecules will be influenced by the overall state of all the other regulatory and metabolic reactions taking place within the cell. Thus, the various signalling and

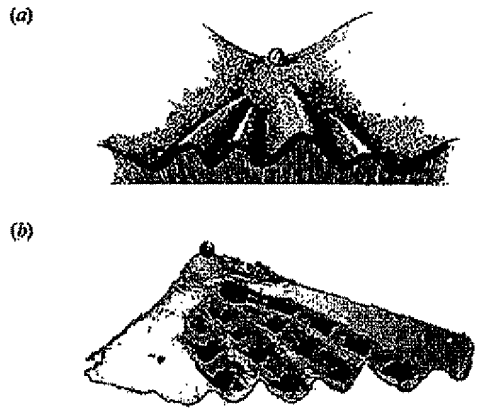

Figure 4. The 'epigenetic landscape': (*a*) a diagram reproduced from a drawing by C. H. Waddington (Waddington 1966) to illustrate his concept of 'canalization'. The image of a ball rolling downhill and selecting between different valleys represents the selection of different lineages by embryonic cells as they differentiate; if something were to push the ball up the side of a valley, it would nevertheless tend to return to the valley bottom, effectively a representation of regulation within the developing embryo. (*b*) A modification of the idea of Waddington: in this image the 'landscape' includes a series of depressions or hollows, which may nevertheless be arranged in valleys. The hollows would represent lowest free energy states, i.e. stable states, of the regulatory/metabolic network that makes up a cell. Movement of a cell from one state to another (differentiation) would tend to be from a stable state of higher free energy to one of lower free energy: the likelihood of such a transition would depend upon the height of the surrounding terrain—low barriers, say between successive hollows in a given valley, would permit a high frequency, or probability, of transition, whereas high barriers, say between neighbouring valleys, would permit transitions only infrequently. Thus, the height of the ground represents something akin to activation energy.

regulatory pathways, as well as the metabolic pathways of a cell, make up a complex, dynamic network in which various 'stable states' or equilibria would probably exist. These stable states, which would correspond to free energy minima for the network, would represent the various differentiated states that a cell could adopt. Under this view, it may be appropriate to modify the 'valleys' envisaged in Waddington's epigenetic landscape to a series of depressions (figure 4*b*), possibly still linked in 'valley systems', that would represent the commonly observed differentiation pathways. The height of the terrain would represent the free energy of the regulatory/metabolic network that constitutes the cells. Therefore the probability of a cell moving from one state to another would depend upon the heights of the hills surrounding the hollows and valleys. Cells finding themselves on the 'uplands' of 'unstable' or 'transition' states would tend to move to the nearest 'stable' state. In such a model, normally observed lineages represent transitions for which there is the highest probability of movement, hence the appearance of irreversibility. However, reversion, or de-differentiation, is entirely possible, although the probability may be low, as would movements from one set of hollows within one valley system to another valley system—trans-differentiation or trans-determination.

Waddington (1962) has indeed discussed the concept of stable states for cells and referred to mathematical models developed by Goodwin (1961). Slack (1991) has also discussed a similar idea. Perhaps a substantial challenge from the recent observations of stem-cell plasticity will be to develop a more detailed understanding of the chemistry underlying the complex network of signalling and metabolic pathways within a cell, to provide a more solid basis for such a concept of 'stable states' as opposed to fixed cell lineages. Certainly, considerable work lies ahead to understand the molecular mechanisms that regulate stem-cell differentiation, before their full potential for regenerative medicine can be realized.

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EXHIBIT 3

Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin

P.W. Andrews*[1], M.M. Matin†, A.R. Bahrami†, I. Damjanov‡, P. Gokhale* and J.S. Draper*

*The Centre for Stem Cell Biology and the Department of Biomedical Science, University of Sheffield, Western Bank, Sheffield S10 2TN, U.K., †Department of Biology, Institute of Biotechnology and Tissue Engineering, Ferdowsi University of Mashhad, Mashhad, Iran, and ‡Department of Pathology, University of Kansas, Kansas City, KS 66160, U.S.A.

Abstract

Embryonal carcinoma (EC) cells are the stem cells of teratocarcinomas, and the malignant counterparts of embryonic stem (ES) cells derived from the inner cell mass of blastocyst-stage embryos, whether human or mouse. On prolonged culture *in vitro*, human ES cells acquire karyotypic changes that are also seen in human EC cells. They also 'adapt', proliferating faster and becoming easier to maintain with time in culture. Furthermore, when cells from such an 'adapted' culture were inoculated into a SCID (severe combined immunodeficient) mouse, we obtained a teratocarcinoma containing histologically recognizable stem cells, which grew out when the tumour was explanted into culture and exhibited properties of the starting ES cells. In these features, the 'adapted' ES cells resembled malignant EC cells. The results suggest that ES cells may develop in culture in ways that mimic changes occurring in EC cells during tumour progression.

Introduction

The concept that cancer arises because of dysfunctional control of stem cells that reside within many adult tissues is an old one. Following the work of Till and McCulloch [1] who identified the existence of stem cell populations capable of regenerating all cell lineages of the blood, stem cell populations have been identified and studied extensively in many other tissues subject to continual renewal throughout life [2,3]. At the same time, the realization by pathologists that tumours often contain the same range of differentiated cell types as the tissue from which they arise, albeit in a disorganized fashion, promoted the idea that aberrant control of differentiation, as much as aberrant control of cell proliferation, lie at the core of oncogenesis [4]. Cairns [5] went on to suggest that stem cells are the targets of carcinogenesis and that a tissue architecture based on a small stem cell population compared with a large population of differentiated cells provides one level of protection against the development of cancer. One consequence of these ideas is that, within tumours, the malignant stem cells are rare and perhaps quiescent, so that they may escape the chemotherapy regimens that destroy most of the cells of a tumour, leaving intact those few cells capable of re-initiating cancer development at a later time.

EC (embryonal carcinoma) cells

Teratocarcinomas, a subset of GCTs (germ cell tumours), provide a striking paradigm of the stem cell concept of cancer. They are highly malignant tumours containing a disorganized array of many somatic and extraembryonic cells, together with nests of EC cells. EC cells are the 'pluripotent' stem cells of these cancers, capable of self-renewal as well as differentiation into a very wide range of cell types. The differentiated derivatives of the EC cells are typically non-malignant, so that malignancy, as shown by the ability to regenerate the whole cancer including its differentiated elements, is the property of the EC stem cells. This was demonstrated by the classic experiments of Kleinsmith and Pierce [6] in which they showed that transplantation of a single EC cell to a new host mouse is sufficient to regenerate a new tumour. However, extensive studies during the 1970s also showed a close relationship between EC cells from murine teratocarcinomas and the pluripotent ICM (inner cell mass) cells of the blastocyst stage of early mouse embryos [7]. This, together with an understanding of how to culture and characterize EC cells *in vitro*, culminated in the isolation of ES (embryonic stem) cell lines by explanting ICM cells from mouse embryos in 1981 [8,9].

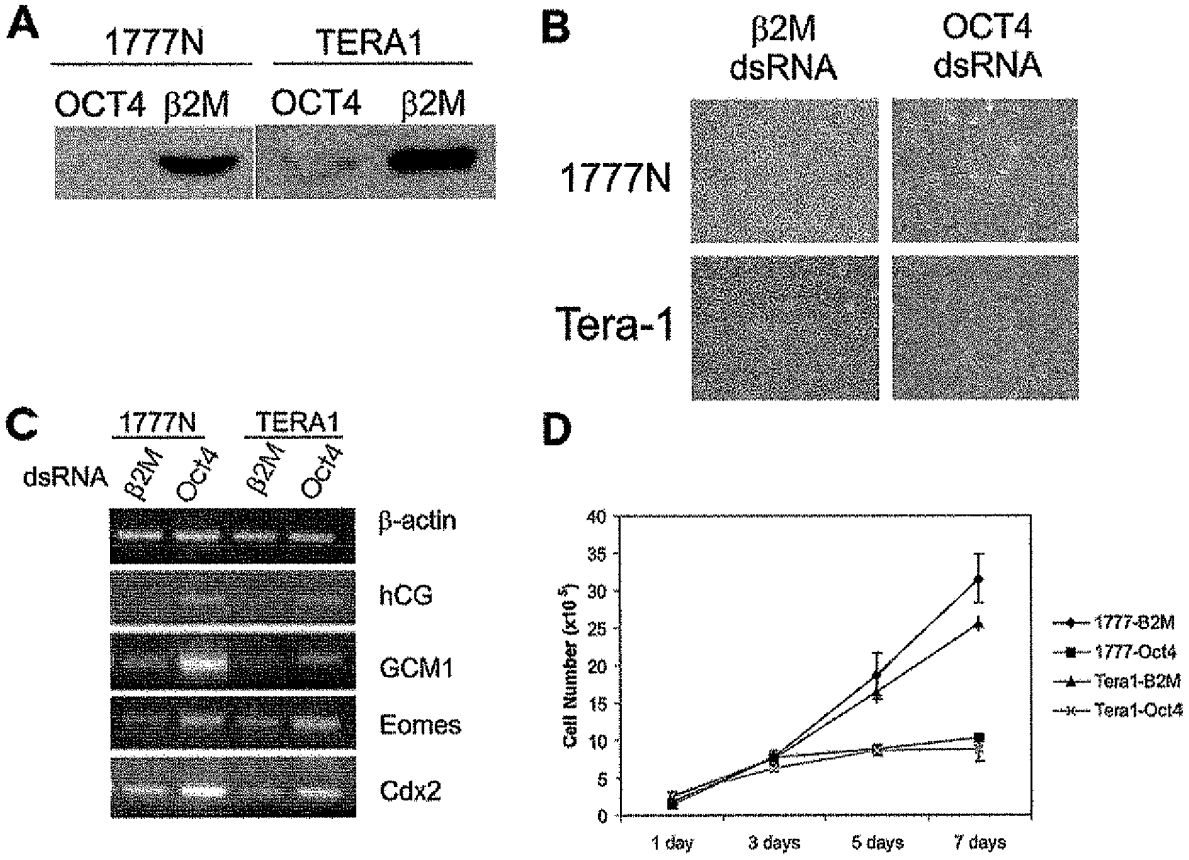
The pathology of human and mouse GCTs is significantly different, while the properties of human and mouse EC cells also differ from one another. For example, while trophoblastic differentiation from human EC cells is common, it does not normally occur from mouse EC cells [10]. Furthermore, they express different patterns of characteristic surface antigens. For example, human EC cells typically express the glycolipid antigens SSEA3 (stage-specific embryonic antigen-3) and SSEA4, but not SSEA1, the high molecular mass proteoglycan antigens TRA-1-60, TRA-1-81 and GCTM2 and the protein antigens Thy1 and MHC class 1; in contrast, murine EC and ES cells express SSEA1 but not the other

Key words: blastocyst, embryonal carcinoma cell, embryonic stem cell, germ cell tumour, pluripotency, teratocarcinoma.

Abbreviations used: EC, embryonal carcinoma; ES, embryonic stem; FGF, fibroblast growth factor; GCT, germ cell tumour; ICM, inner cell mass; RNAi, RNA interference.

[1]To whom correspondence should be addressed (email p.w.andrews@sheffield.ac.uk).

Figure 1 | Knockdown of *OCT4* expression by RNAi in human EC cells results in differentiation and growth arrest
Two human EC cell lines, 1777N and TERA-1, were transfected with double-stranded RNA (dsRNA) corresponding to either *OCT4* or β_2 microglobulin (*β2M*), as a negative control, as described previously [16]. (**A**) Western blot for *OCT4* expression in cells 5 days after treating with siRNA (small interfering RNA) targeting either *β2M* or *OCT4*. Note the substantial reduction in *OCT4* after treatment with *OCT4* siRNA compared with the level in cells treated with an siRNA targeting *β2M*. The lanes were each loaded with a lysate containing the equivalent of 1.5×10^5 cells. (**B**) Photomicrographs of 1777N and TERA-1 EC cells 4 days after *β2M* (left panels) or *OCT4* (right panels) RNAi. (**C**) Reverse transcriptase-PCR for the trophoblast-specific transcripts *hCG* (human chorionic gonadotrophin), *GCM1* (glial cells missing 1), *Eomes* and *Cdx2* after treatment with siRNA to *OCT4*. Note the induction of each gene after *OCT4* knockdown. (**D**) Effect of *OCT4* knockdown on the population growth rate after treatment with β2M and *OCT4* siRNAs ($n = 3$; ±S.D.).



markers [11]. It is noteworthy, then, that when human ES cells were finally derived by explanting the ICM of human blastocysts, their properties closely paralleled those of human EC cells and were distinct from those of mouse ES cells [12,13]. Human ICM cells from blastocysts also express similar patterns of surface antigen expression to both human EC and ES cells, again confirming the relationships between these cell types and emphasizing that the differences from the corresponding mouse cells most probably represent species differences in embryogenesis [14].

Nevertheless, like murine EC and ES cells, human EC and ES cells characteristically express the transcription factor OCT4, which is down-regulated upon differentiation. Moreover, as in human and mouse ES cells [15,16], knockdown of *OCT4* expression in human EC cells using RNAi (RNA interference) techniques results in their differentiation towards trophectoderm (Figure 1). Since *OCT4* knockdown also results in a substantial reduction, if not elimination, of cell growth, this observation also suggests a potential therapeutic target for RNAi if an appropriate delivery technology could be developed.

Adaptation and tumour progression

Despite their similarities, teratocarcinoma-derived EC cells present only a caricature of ES cells. In contrast with ES cells, EC cells often only have a limited capacity for differentiation, and many EC cell lines have completely lost this ability – such cell lines are called 'nullipotent'. Many, particularly in humans, are also karyotypically abnormal [17].

Such differences from ES cells are not particularly surprising when it is appreciated that EC cells have necessarily been selected for tumour growth. While many facets of cell biology might contribute to better survival of tumour cells, a particular feature that is pertinent in the case of pluripotent stem cells is their capacity to choose between the production of daughter stem cells (self-renewal) on the one hand, and differentiation on the other. Since the differentiated derivatives of EC cells have limited competence for extended proliferation and survival, one might infer that pluripotent stem cells like EC cells will be subject to strong selection for mutations that tend to limit differentiation – even a small increase in the probability of self-renewal compared with differentiation could have a significant selective advantage during tumour progression.

If indeed the selection of variants with reduced capacity for differentiation is to be expected in the growth of EC cells, similar selection might also be anticipated in ES cells on prolonged passage in culture. Recently, we have shown that human ES cells in culture commonly acquire additional copies of chromosome 17, particularly its long arm (17q) and also chromosome 12, particularly its short arm (12p) [18]. What is striking about this observation is that the same chromosomal additions are very common in EC cells from human teratocarcinomas [19–21]. Furthermore, amplification of mouse chromosome 11, which is syntenic with much of human chromosome 17q, has also been reported in mouse ES cells [22], although mouse chromosome 8 is also commonly amplified too. One inference from these observations is that additional copies of a gene(s) encoded by these chromosomes contribute to enhancing the capacity of both EC and ES cells for self-renewal and that such genes encode key components of the molecular mechanism by which these stem cells choose whether or not to commit to differentiation. To date, the identity of these genes remains unknown, although some studies in EC cells have focused on *GRB7* as a potential candidate gene that is frequently highly expressed in human EC cells and is located on chromosome 17q [20].

To explore the issue of culture adaptation of human ES cells further, we have particularly studied a 'culture adapted' subline of H7 human ES cells. This cell line originally had a normal human 46,XX karyotype. However, the adapted subline (H7-s6), which shows substantially enhanced growth characteristics, had acquired a translocation of the long arm of chromosome 17 to chromosome 6 [46,XX, der(6)t(6;17)(q27;q1)]; effectively these cells were trisomic for chromosome 17q [18]. Subsequently, on further passage, we have found that the cells acquired an extra copy of chromosome 1, so that their karyotype became 47,XX,+1, der(6)t(6;17)(q27;q1). Generally, human ES cells are cultured in the presence of inactivated feeder cells or in the presence of conditioned medium from such cells. However, from H7-s6, we developed further sublines that retained a pluripotent phenotype even when cultured for a long period without feeders, or conditioned medium, albeit on a Matrigel substrate [23]. One of these sublines was also adapted to grow without feeders and in the absence of FGF (fibroblast growth factor), a growth factor generally thought to be required by human ES cells. Nevertheless, these cells retained pluripotency, expressing typical markers of human ES cells [23] and produced teratomas when grown in immunosuppressed mice (Figure 2).

One tumour derived from H7-s6 cells also contained cells with the morphological appearance of undifferentiated stem cells – indeed, they resembled EC cells in teratocarcinomas (Figure 2). Furthermore, when this tumour was explanted in culture, cells grew out with the general features of human ES cells, such as the morphology and expression of marker antigens such as SSEA3 and TRA-1-60 (Figure 2). Thus progressive adaptation of human ES cells in culture can result in cells with all the hallmarks of EC cells from teratocarcinomas, while still retaining the capacity for differentiation. The cells in the explanted culture had the same karyotypic changes as the cells injected to form the tumour except that approx. 75% had also acquired an additional copy of chromosome 8 [48,XX,+1,der(6)t(6;17)(q27;q1),+8].

Concluding comments

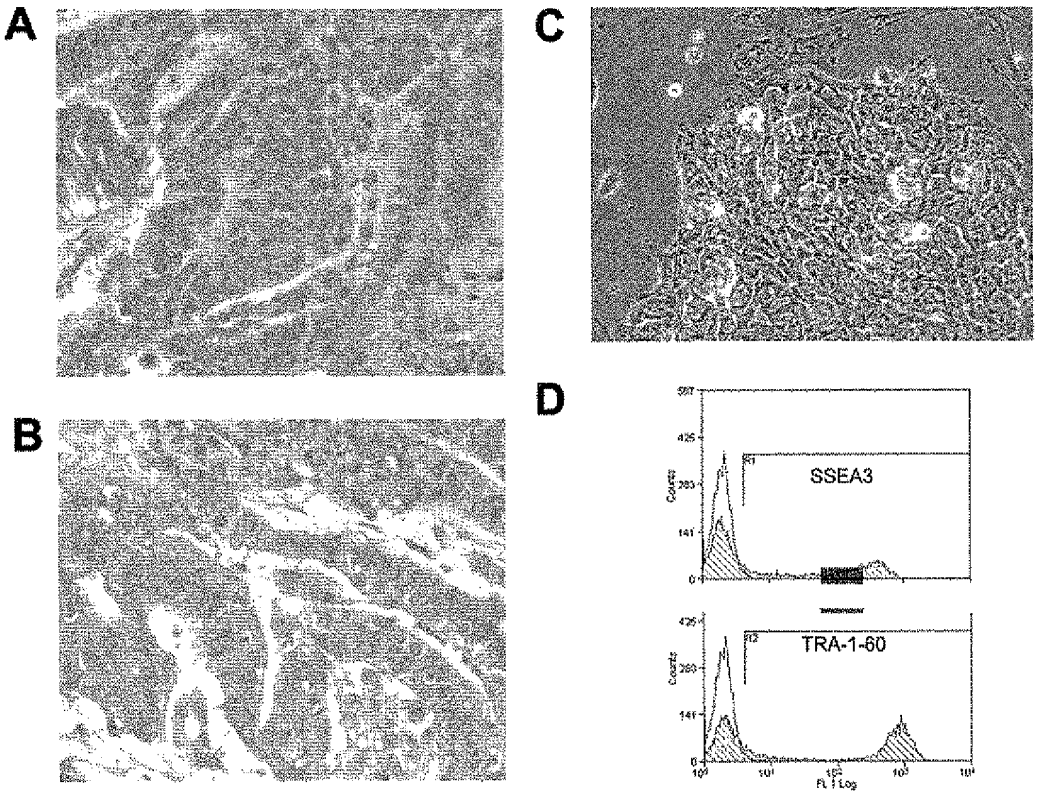
Adaptation of human ES cells after prolonged culture is clearly not surprising. The moment an ICM cell is explanted *in vitro* it is exposed to a new environment. Its capacity for indefinite proliferation, contrasting with the transitory nature of ICM cells *in vivo*, is certainly one indication of change. Evidently, any initial change that adapts the cells to *in vitro* culture is readily reversible, at least in the mouse, since mouse ES cells can contribute to all cell types of a developing embryo, including its germline, when introduced into a blastocyst. But even among mouse ES cells, this capacity may be lost with time, associated with progressive aneuploidy [24].

In the light of these observations, EC cells from teratocarcinomas and ES cells from embryos might be regarded as existing at different points along a continuous spectrum of adaptation, from complete 'normality' at one end (represented by the ICM cell within an embryo) to extreme 'abnormality' at the other (represented by a nullipotent EC cell from a GCT). Thought of in this way, ES and EC cells may provide information that is pertinent one to the other, and ES and EC cell lines can provide complementary tools for exploring problems of pluripotency, stem cell biology and cancer. However, the ability of ES cells to undergo progressive adaptation sounds one particular note of caution in relation to experiments to identify key factors that drive their self-renewal. It is quite possible that the cells will have different culture requirements at different stages along the adaptation spectrum – from a requirement for feeders at one end to complete feeder independence, and probably minimal extrinsic factor requirements, at the other. Indeed, as we have found, various sublines of a human ES cell can be isolated with different requirements for feeders and FGF while yet retaining many key features of pluripotent ES cells.

The practical significance for the eventual therapeutic applications of ES cells of this relationship between ES and EC cells, and the phenomenon of adaptation in culture,

Figure 2 | Xenograft tumours derived from a 'culture adapted' human ES cell line

(**A**) Small foetal neural cells forming neural tubes and rosettes from a tumour derived from a cloned human ES cell subline of H7 cells (H7.5A.cl.F1), adapted to grow *in vitro* without feeders; haematoxylin and eosin, ×220. (**B**) Undifferentiated cells, corresponding to undifferentiated ES cells forming irregular cords from a tumour of culture-adapted H7-s6 cells; haematoxylin and eosin, ×220. (**C**) ES-like cells growing out *in vitro* from the H7-s6 tumour shown in (**B**). (**D**) Fluorescence histograms of cells from (**C**) analysed by flow cytofluorimetry for the ES cell marker antigens TRA-1-60 and SSEA3. The histograms represent the negative controls; the hatched histograms show cells reacting with the specific antibodies: 56% TRA-1-60(+), 43% SSEA3(+); the antigen-negative cells are most probably differentiated derivatives of the ES cells.



should not be overstated. It is certain that our current culture techniques are suboptimal. Future developments based upon a firm understanding of the biology of human ES cells and the molecular mechanisms that drive self-renewal and commitment to differentiation will undoubtedly lead to culture methods that will minimize the selective advantage of variant cells. Furthermore, a genetic variant that offers a selective advantage to an undifferentiated ES cell in culture might have no significance for the behaviour and function of a differentiated derivative, although this would clearly need to be demonstrated in any particular case.

However, the phenomenon of adaptation may also be put to good use. 'Adapted' ES cells that retain pluripotency may be simpler to use in practical applications such as high-throughput drug screening. More importantly, they may also give us insights into the mechanisms that are involved in controlling stem cell self-renewal, and commitment to differentiation. Finally, since the typical karyotypic changes in adapted ES cells mirror those found in their malignant counterparts from teratocarcinomas, the mechanisms that drive adaptation and selection of such variants *in vitro* may give insights into the mechanisms underlying GCT progression *in vivo*. In GCT, the cells are typically grossly aneuploid with many karyotypic changes, many of them being case-specific – only a few of the changes are common to all cases of the tumour and so likely to be relevant to the general mechanisms of self-renewal and differentiation. The relatively few karyotypic changes occurring in adapted ES cells might make analysis of the underlying causes simpler.

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REVIEW

Surface antigens of human embryonic stem cells: changes upon differentiation in culture*

Jonathan S. Draper,[1] Christine Pigott,[1] James A. Thomson[2] and Peter W. Andrews[1]

[1]Department of Biomedical Science, University of Sheffield, Sheffield S10 2TN, UK
[2]University of Madison at Wisconsin, Wisconsin Regional Primate Research Centre and Department of Anatomy, School of Medicine, University of Wisconsin, Madison, WI 53715, USA

Abstract

We have analysed the surface antigen phenotype of a human embryonic stem (hES) cell line (H7) and the changes that occur upon differentiation induced by retinoic acid, hexamethylene bisacetamide and dimethylsulphoxide. The undifferentiated stem cells expressed Stage Specific Embryonic Antigen-3 (SSEA3), SSEA4, TRA-1-60, and TRA-1-8 but not SSEA1. In these characteristics they closely resemble human embryonal carcinoma (EC) cells derived from testicular teratocarcinomas, and are distinct from murine EC and ES cells. The undifferentiated cells also expressed the liver/bone/kidney isozyme of alkaline phosphatase detected by antibody TRA-2-54, the class 1 major histocompatability antigens, HLA-ABC, and the human Thy1 antigen. Differentiation of hES cells was induced by retinoic acid, HMBA and DMSO with the appearance of various cell types including neurons and muscle cells. The surface antigens characteristically expressed by hES cells were down-regulated following induction of differentiation and other antigens appeared, notably several ganglioside glycolipids detected by antibodies VIN-IS-56 (GD3 and GD2), VIN-2PB-22 (GD2), A2B5 (GT3) and ME311 (9-O-acetyl-GD3). Whereas the expression of HLA was slightly down-regulated upon differentiation, its expression was strongly induced by interferon-γ in both the undifferentiated and the differentiated cells, although the induction in the differentiated cultures was considerably stronger than in the stem cells. In all of these features the human ES cells, and their pattern of differentiation, resembled the pluripotent human EC cell line NTERA-2 although clearly the range of cells generated by the hES cells was considerably greater.

Key words stem cells; surface antigens.

Introduction

EC cells are the malignant stem cells of teratocarcinomas, a subset of germ cell tumours that may contain many embryonic and extra-embryonic tissues, and apparently recapitulate many aspects of cell differentiation that occur during normal embryogenesis, albeit in a disorganized manner. Both spontaneous and experimentally induced teratocarcinomas occur in the laboratory mouse (Solter & Damjanov, 1979). By now it is well documented that murine EC cells isolated from such teratocarcinomas closely resemble cells of the inner cell mass from the blastocyst stage of the early mouse embryo, and embryonic stem (ES) cells cultured directly from explanted blastocysts (Martin, 1980). For example, when mouse EC and ES cells are placed into a blastocyst they can participate in embryonic development, and contribute to normal tissues in the resulting chimeric embryo (Brinster, 1974; Papaioannou et al. 1975). ES cells form chimeras much more efficiently than EC cells, but this may well reflect the accumulation of genetic and epigenetic changes that occur in EC cells as they adapt to efficient tumour growth.

Correspondence
Peter W. Andrews, Department of Biomedical Science, University of Sheffield, Western Bank, Sheffield S10 2TN, UK.
Tel.: +44 (0)114 2224173; fax: +44 (0)114 2222399;
e-mail: P.W.Andrews@Sheffield.ac.uk

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Until the recent derivation of human ES cell lines, human EC cell lines provided the best model for studying cell differentiation pertinent to human embryonic development (Andrews, 1998). For example, the human teratocarcinoma-derived cell line NTERA2 is composed of pluripotent EC cells that differentiate in response to retinoic acid, yielding a variety of cell types including functional, post-mitotic neurons (Andrews et al. 1984b; Andrews, 1984; Rendt et al. 1989; Pleasure et al. 1992; Squires et al. 1996). This differentiation is characterized by marked changes in gene expression (Ackerman et al. 1994) but most notably by the activation of HOX genes in a retinoic acid concentration-dependent manner (Simeone et al. 1990). However, changes in the expression of cell surface antigens provide a particularly powerful means of monitoring differentiation, not only because antigen expression can be readily assessed on single cells in complex differentiating populations, but because subsets of viable cells corresponding to different stages of differentiation, or different lineages, can be isolated in order to analyse their individual properties and capacity for further differentiation (Fenderson et al. 1987).

Human EC cells, like NTERA2, are characterized by expression of the globoseries glycolipid antigens SSEA3 and SSEA4, and by a lack expression of a lactoseries oligosaccharide antigen, SSEA1 (Andrews et al. 1982, 1984b, 1996). They also express the keratan sulphate-related antigens, TRA-1-60 and TRA-1-81 (Andrews et al. 1984a; Badcock et al. 1999) and the tissue non-specific alkaline phosphatase-related antigens TRA-2-49 and TRA-2-54 (Andrews et al. 1984c). Differentiation induced by retinoic acid is marked by the down-regulation of all of these antigens, and by the appearance of other antigens including SSEA1 and several ganglioseries glycolipid antigens, which segregate on different subsets of cells (Fenderson et al. 1987). If differentiation is induced by another agent, hexamethylene bisacetamide (HMBA), the EC-specific antigens are similarly down-regulated, but the antigens induced by retinoic acid do not appear, consistent with the notion that retinoic acid and HMBA induce differentiation along distinct lineages (Andrews et al. 1990).

The recent derivation of ES cell lines from human embryos (Thomson et al. 1998; Reubinoff et al. 2000) now provides additional tools for investigating the molecular processes that guide human development and the causes of infertility and birth defects when these mechanisms operate incorrectly. Further, human ES cells could provide a potential source of 'normal' differentiated cell types for transplantation therapies and drug discovery.

Initial studies have confirmed the anticipated similarities between human ES and EC cells, and significant differences in antigen expression and other properties between human ES and EC cells and their murine counterparts (Andrews, 1998). We have now examined the changes in surface antigen expression that occur during the differentiation of human ES cells induced by retinoic acid, HMBA or DMSO, or by culture in the absence of feeders. As anticipated, a number of changes that occur during EC cell differentiation, notably down-regulation of stem-cell-specific markers, also occurred during the differentiation of ES cells. There were also some differences in the patterns of antigens induced, mostly likely because of the greater diversity of differentiated cell types that appear during ES cell differentiation.

Materials and methods

Cell culture

The H7 human ES (hES) cell line, described by Thomson et al. (1998), was cultured on mouse embryonic fibroblast (MEF) feeder cells, mitotically inactivated using Mitomycin-C, as previously described. However, the cells were cultured in DMEM-SR medium (Gibco-BRL) supplemented with 4 ng mL^{-1} bFGF (Gibco-BRL) and 20% 'Serum Replacement' (SR) (Gibco-BRL) instead of fetal calf serum. Briefly, MEFs isolated from 13-day mouse embryos (Robertson, 1987) were treated with 10 $\mu g\ mL^{-1}$ mitomycin-C (Sigma Aldrich) for 2.5 h. Subsequently, the treated cells were washed with PBS, harvested with trypsin-EDTA and reseeded at 10^4 cells per cm^2 on gelatin-treated tissue culture dishes. For passaging, the H7 hES cells were treated with 1 mg mL^{-1} Collagenase type IV (Sigma Aldrich) in DMEM:F12 for 8–10 min at 37 °C and then detached by scraping using glass beads (Andrews et al. 1984b), washed by centrifugation, and re-plated onto inactivated MEFs. In some experiments, H7 hES cells were plated on tissue culture dishes that had been pretreated with poly D-lysine and Matrigel (Becton-Dickinson) in the absence of inactivated MEFs. Differentiation was induced by adding 10^{-5} M all *trans*-retinoic acid (RA) (Eastman Kodak), 3 mM hexamethylene bisacetamide (HMBA) (Sigma Aldrich), or 1% dimethylsulphoxide (DMSO) as described by Andrews

(1984), Andrews et al. (1990) and Andrews et al. (1986), respectively. Human Interferon-γ (IFNγ) was purchased from Sigma Aldrich.

NTERA2 cL.D1 human EC cells (Andrews et al. 1984b) were cultured as previously described and induced to differentiate by treatment with 10^{-5} M all-*trans* retinoic acid (RA) (Eastman Kodak) (Andrews, 1984).

Surface antigen expression

Cell surface antigen expression was assessed by immunofluorescence detected by flow cytofluorimetry after harvesting cultures as single cell suspensions using trypsin-EDTA, as previously described (Andrews et al. 1987a, 1987b; Fenderson et al. 1987). The following monoclonal antibodies were used to detect surface antigen expression: MC631, anti-Stage Specific Embryonic Antigen-3 (SSEA3) (Shevinsky et al. 1982), MC813-70, anti-Stage Specific Embryonic Antigen-4 (SSEA4) (Kannagi et al. 1983), MC480, anti-Stage Specific Embryonic Antigen-1 (SSEA1) (Solter & Knowles, 1978), TRA-1-60 and TRA-1-81 (Andrews et al. 1984a), TRA-2-54, anti-liver/kidney/bone alkaline phosphatase (Andrews et al. 1984c), A2B5 (Eisenbarth et al. 1979; Fenderson et al. 1987), ME311 (Thurin et al. 1985; Fenderson et al. 1987), VIN-IS-56 and VIN-2B-22 (Andrews et al. 1990), N901 (Griffin et al. 1983) and anti-Thy1 (McKenzie & Fabre, 1981), W6/32 (Barnstable et al. 1978), BBM1 (Brodsky et al. 1979). TRA-1-85 (Williams et al. 1988), which detects a pan-human antigen, was used to monitor contamination with mouse feeder cells.

Results

Cultures of H7 hES cells grown on mouse embryo fibroblast feeders contained many colonies of cells that closely resembled human EC cells (Fig. 1). If the cells were plated onto tissue culture dishes precoated with poly D-lysine and Matrigel, but without MEFs, many of the cells appeared to differentiate and many colonies of cells with markedly different morphologies appeared, although occasional colonies of cells retaining an ES phenotype persisted. If all-*trans*-retinoic acid was included into the cultures, either grown on feeder layers, or after plating on Matrigel, even more marked differentiation occurred and a variety of cell types, for example neurons, glia and muscle cells, could be detected by immunofluorescence staining with appropriate antibodies (Fig. 2).

We then examined the expression of a panel of antigens previously analysed on human EC cells (Fig. 3). As anticipated from previous reports, the hES cells from stock cultures, at the time of reseeding, strongly expressed the markers that also characterize undifferentiated EC cells, namely SSEA3, SSEA4, TRA-1-60 and TRA-1-81. They also strongly expressed Thy1, and the liver/bone/kidney isozyme of alkaline phosphatase detected by reactivity with antibody TRA-2-54. By contrast, they expressed relatively low levels of SSEA1. These characteristics are typical of the surface antigen phenotype of human EC cells and distinct in certain respects from murine EC cells. The proportion of feeder cells at the time of assay, monitored by reactivity with

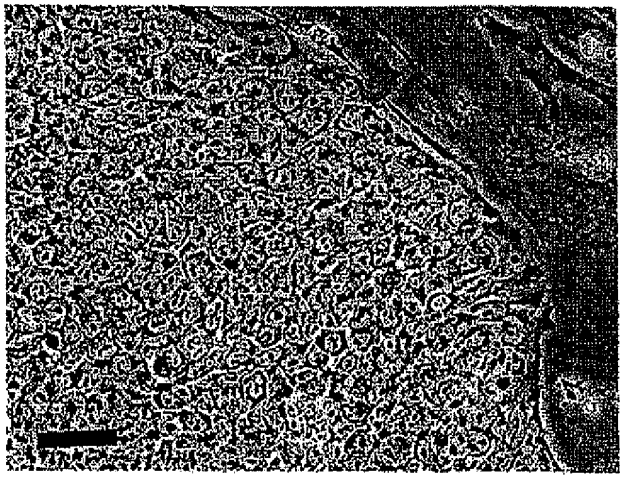

Fig. 1 Phase contrast photomicrograph of an undifferentiated human ES cell colony. The cells resembled human EC cells morphologically, forming tight colonies composed of cells with high nucleus/cytoplasm ratios and containing few prominent nucleoli. Scale bar = 50 μm.

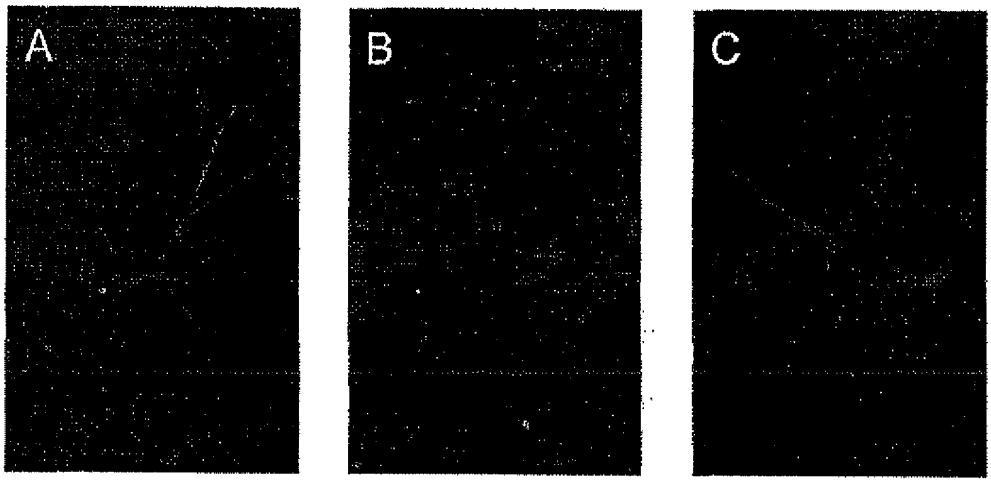

Fig. 2 Neurons, glial and muscle cells detected by immunofluorescence with antibodies to neurofilaments (A), GFAP (B) and desmin (C) were found in the hES H7 cultures grown in the presence of 10^{-6} M retinoic acid.

an antibody to a pan-human antigen, TRA-1-85, was negligible (< 2%) (data not shown).

During subsequent culture, expression of SSEA3, SSEA4, TRA-1-60, TRA-1-81, TRA-2-54 and Thy1 remained high when the ES cells were cultured on MEFs in the absence of retinoic acid. However, these antigens were down-regulated when the cells were plated on Matrigel in the absence of MEFs, or when they were cultured in the presence of retinoic acid, whether on MEFs or on Matrigel. Such changes are consistent with the differentiation of the cells, as suggested by morphological examination, and are comparable to changes seen during the differentiation of human EC cells.

The kinetics of disappearance of these antigens differed from one another. In particular, SSEA3 disappeared more quickly than SSEA4, a result which we have consistently found before in the case of differentiating human EC cells (Fenderson et al. 1987). TRA-1-60 and TRA-1-81 also disappeared rapidly, whereas TRA-2-54 and Thy1 tended to disappear relatively slowly. Whether this reflects persistent expression on a subset of differentiating cells or a slower turnover of the antigens was not assessed. A further notable point was that although the EC/ES markers were down-regulated when the cells were cultured on Matrigel in the absence of retinoic acid, a significant proportion of cells that continued expressing these antigens nevertheless persisted, suggesting that at least during short-term culture the presence of MEFs is not an absolute requirement for maintenance of an undifferentiated phenotype.

Differentiation of the NTERA2 EC cell line is marked by transient up-regulation of SSEA1 and a strong up-regulation of gangliosides, notably GT3 detected by A2B5, all consistent with a marked propensity of these cells to differentiate in a neuroectodermal direction (Fenderson et al. 1987). In the case of hES cells, the gangliosides GD3 and GD2 detected by antibodies VIN-IS-56 and VIN-2PB-22 were also up-regulated when the cells were cultured either without feeders or after addition of retinoic acid, but only relatively small numbers of cells expressed antigens detected by A2B5 (GT3) and ME311 (9-O-acetyl-GD3). In fact the greatest induction of these antigens appeared to occur without retinoic acid, when the cells were plated on Matrigel. SSEA1(+) cells were seen after culture on Matrigel and, even more after the addition of retinoic acid, although their numbers subsequently declined. The antigen detected by antibody N901, CD56, a version of NCAM, was up-regulated after addition of retinoic acid and to some extent after culture on Matrigel in the absence of retinoic acid.

The presence or absence of bFGF in the medium during differentiation had no significant effect on the pattern of antigen expression (data not shown).

The class 1 Major Histocompatibility Complex (MHC) antigens (HLA) are commonly expressed by human EC cells, in contrast to their absence from murine EC and

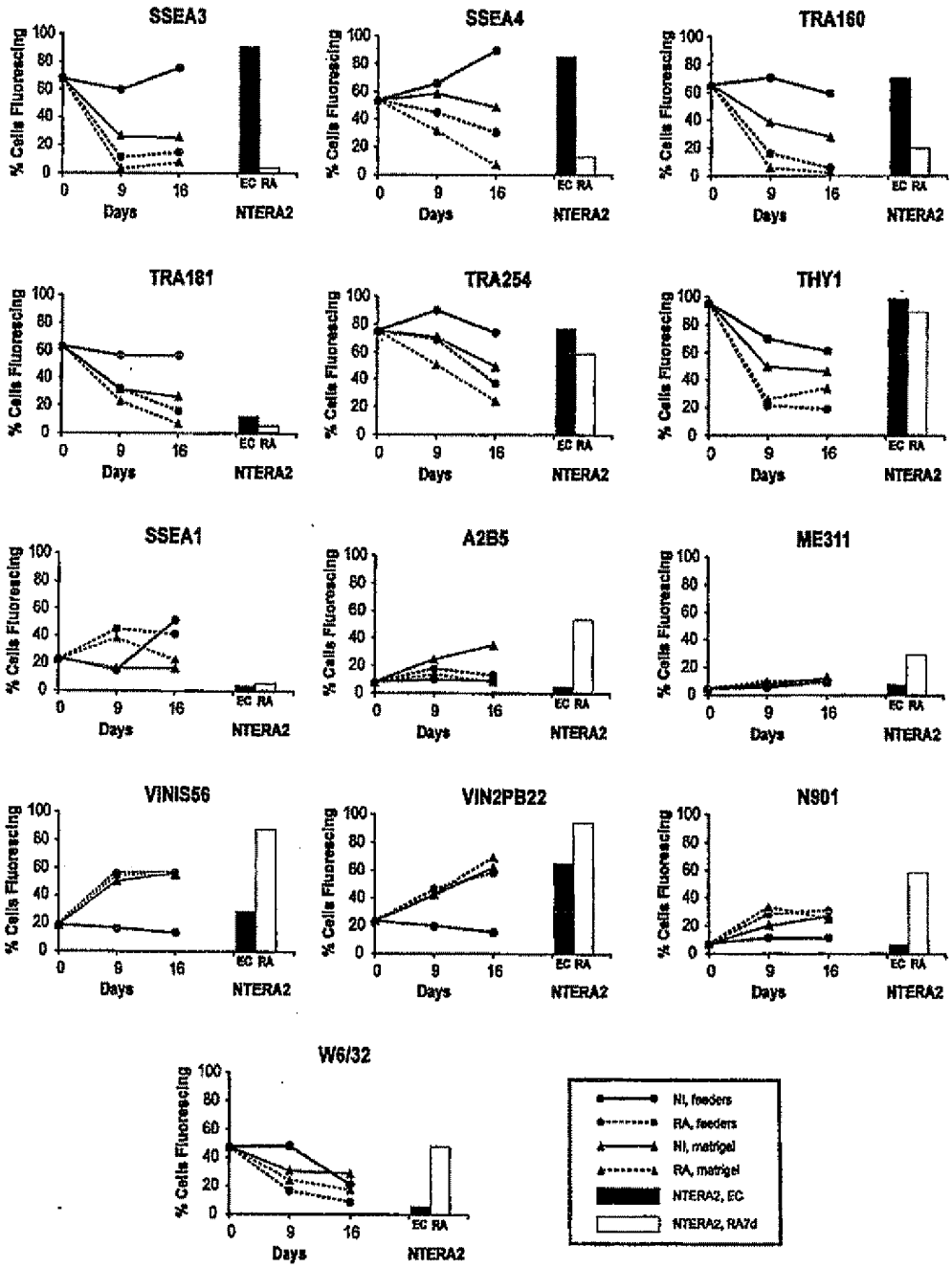

Fig. 3 Changes in surface antigen expression by hES H7 cells when cultured on MEF feeders or Matrigel, in the presence or absence of 10^{-5} M retinoic acid. For comparison typical antigen expression by NTERA2 EC cells and NTERA2 cells induced to differentiate with retinoic acid for 7 days are also shown.

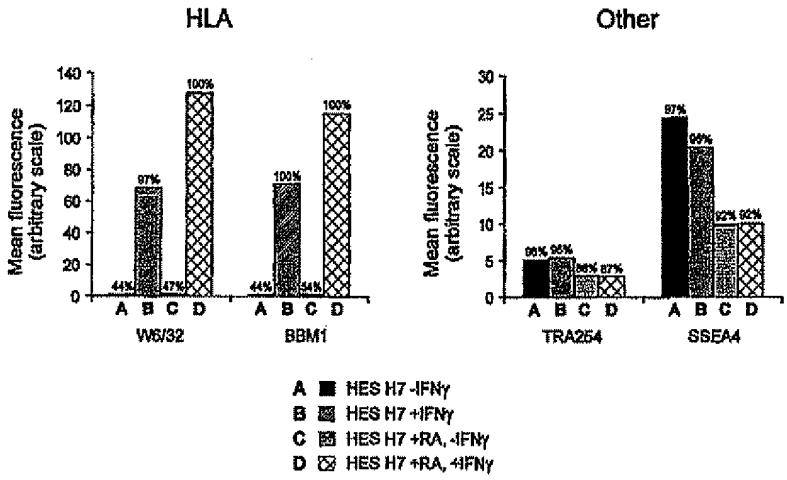

Fig. 4 The effect of IFNγ on the expression of HLA (HLA-A,B,C and β_2 microglobulin) and the antigens (alkaline phosphatase and SSEA4) by hES H7 cells. The histograms show the mean fluorescence intensity of cells after staining with antibodies W6/32 (anti HLA-A,B,C), BBM1 (anti-β_2 microglobulin), TRA-2-54 (anti-liver/bone/kidney alkaline phosphatase) and MCX813-70 (anti-SSEA4); the percentage of cells fluorescing is indicated above each bar.

ES cells (Andrews, 1998). HLA-A, B, C, the class 1 MHC antigen detected by antibody W6/32, was expressed by the undifferentiated H7 hES cells (Fig. 3), and some down-regulation was noted after differentiation induced with retinoic acid or on Matrigel or indeed on extended cultures. In human EC cells, HLA is strongly inducible by IFNγ without any obvious effects on differentiation, and is inducible to a higher level after differentiation. We have now found that the same is true of the H7 hES cells (Fig. 4). Thus, HLA-A, B, C, detected by W6/32, and β2 microglobulin, detected by antibody BBM1, were both much more strongly expressed after culture of the cells for 3 days in the presence of IFNγ. However, considerably stronger expression was noted on cells that had been pretreated with retinoic acid. The effect was specific to HLA; the expression of other surface antigens, for example, TRA-2-54 and SSEA4, was not affected by culture in the presence of IFNγ suggesting that there was no effect on the state of differentiation of the cells.

Although retinoic acid is, perhaps, the most widely used agent to induce differentiation of EC cells and ES cells, in both the mouse and human, other agents are also known to cause undifferentiated pluripotent cells to differentiate. Notably, HMBA induces differentiation of mouse (Jakob et al. 1978) and human EC cells (Andrews et al. 1990), while DMSO which induces differentiation of mouse EC cells (McBurney et al. 1982) has very little effect on, at least, the NTERA-2 human EC cell line (Andrews et al. 1986). When H7 hES cells were cultured in the presence of 1% DMSO or 3 mM HMBA (Fig. 5) after plating on Matrigel, without MEFs, significant differentiation was indicated by changes in antigen expression. All the antigens characteristic of human EC and ES cells, SSEA3, SSEA4, TRA-1-60, TRA-1-81, TRA 2-54 and Thy1, were strongly down-regulated in cultures exposed to the DMSO and HMBA. Again, SSEA3 disappeared rather more rapidly than SSEA4. At the same time antigens detected by antibodies A2B5, ME311 and N901 were also all induced by both DMSO and HMBA. Indeed, A2B5 reactivity appeared to be induced to a greater extent by DMSO than by retinoic acid. Neither agent induced SSEA1 to any significant extent. Thus, as with EC cells, HMBA and DMSO appear to induce differentiation along lineages to some extent distinct from those induced by retinoic acid.

Discussion

It has not always been evident that EC and ES cells in humans are related cell types. Although in the laboratory mouse EC cells do closely resemble ES cells, and may be considered their malignant counterparts, human EC cells differ in many respects from murine EC and ES cells. Thus the cell surface antigen phenotype of human EC cells is typically SSEA1(–) but SSEA3(+) and

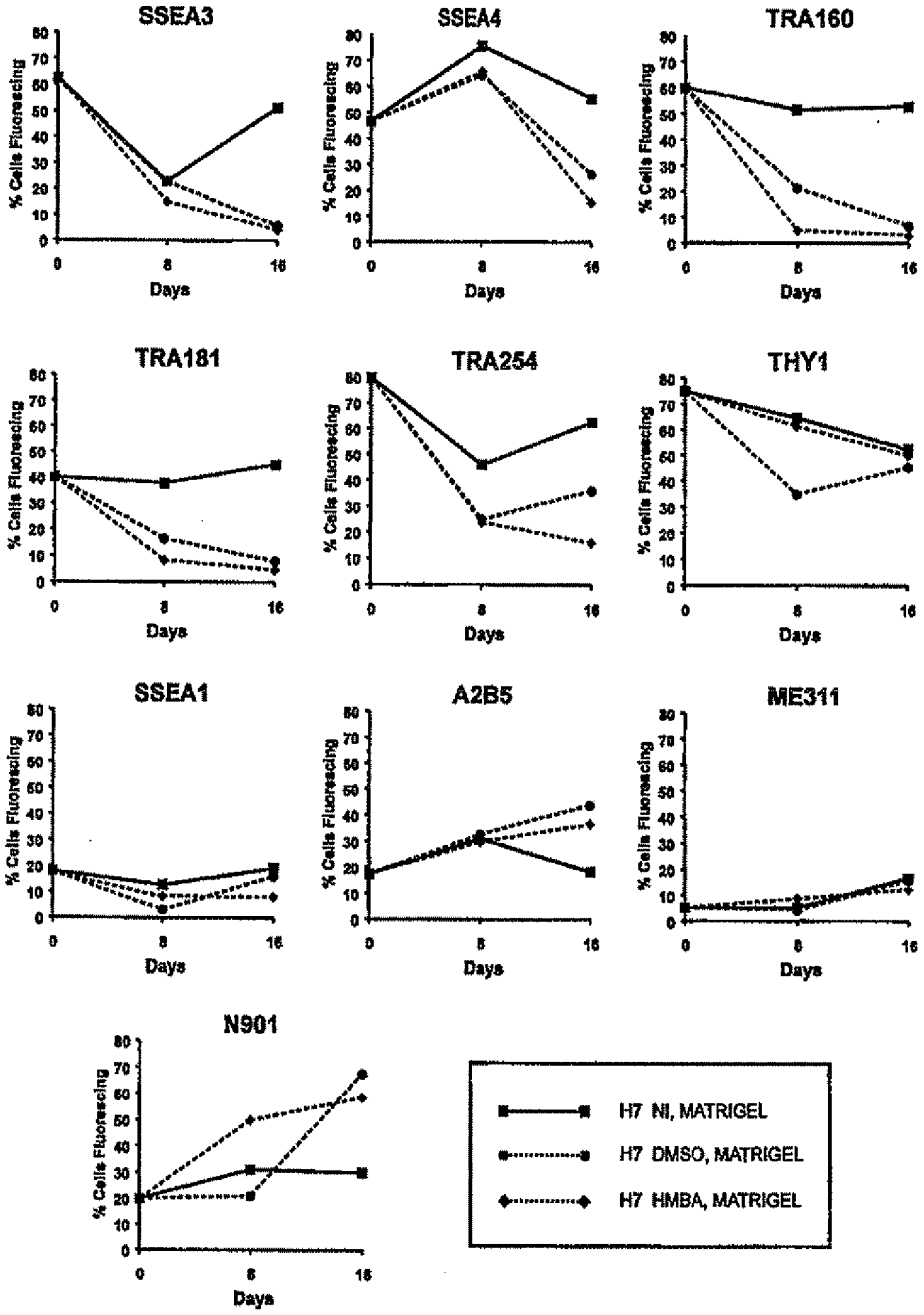

Fig. 5 Surface antigen expression by hES. H7 cells were cultured on Matrigel in the presence, or absence, of 3 mM HMBA or 1% (v/v) DMSO.

SSEA4(+), differing from that of murine EC cells, which are typically SSEA1(+) but SSEA3(–) and SSEA4(–) (Andrews, 1998). A further difference is the definite but low level expression of the class 1 MHC antigens by human EC cells, but not by mouse EC cells (Andrews et al. 1981, 1982, 1984b, 1996). Because of these differences from mouse EC and ES cells it was unclear whether human ES cells derived from human embryos would prove distinct from human EC cells from teratocarcinomas, or whether human ES and EC cells would resemble one another, so that the differences from the mouse would represent differences between the embryos of the two species.

Recent reports, as well as the present study, confirm the similarity of human EC and ES cells, and the existence of differences between comparable cells of humans and mice. Thus, human EC and ES cells are both characterized by their expression of SSEA3, SSEA4, TRA-1-60, TRA-1-81 antigens, as well as the liver/bone/kidney isozyme of alkaline phosphatase (detected by TRA-2-54) and human Thy1 antigen. We have now also demonstrated that these antigens are down-regulated during the differentiation of human ES cells, and that several antigens induced during the differentiation of human EC cells are also induced during the differentiation of ES cells. On the other hand, there are some differences. Whereas several gangliosides are induced during differentiation of human ES cells as detected by antibodies VIN-15-56 and VIN-2B8-22 (GD3 and GD2), other ganglioside antigens that are strongly expressed during NTERA2 human EC differentiation, GT3 detecting A2B5 and 9-O-acetyl GD3 detecting ME311, were only induced to relatively low levels. Most likely, this reflects rather more heterogeneity within the differentiating human ES cultures. The marked ganglioside expression is most likely particularly associated with neuroectodermal differentiation which occurs in both hES and NTERA2 cultures. However, whereas the hES cells clearly generate muscle and mesodermal derivatives, there is no evidence that NTERA2 is capable of mesodermal differentiation (Gokhale et al. 2000)

The expression of HLA by the human ES cells and its tendency for slight down-regulation upon differentiation is another feature that they share in common with human EC cells. In both cases HLA is strongly induced by IFNγ while amongst the differentiated cultures, the induction of HLA by IFNγ is considerably greater than in the undifferentiated cells. Indeed, IFNγ can also induce the expression of MHC antigens in some mouse EC cells, even though expression is generally not detectable in the absence of this inducer. In both murine and human EC cells it has been shown that these cells only exhibit a partial response to IFNγ without a fully fledged antiviral response (Andrews et al. 1987).

Surface antigen markers provide invaluable tools for monitoring the progress of differentiation and isolate subsets of cells to explore their functions and capacity for further differentiation. Many of the antigens we have studied are associated with carbohydrate epitopes, some linked with glycolipids (e.g. the SSEA series), some with glycoproteins (TRA-1-60 and TRA-1-81). Others are associated with protein epitopes, notably TRA-2-54 (alkaline phosphatase), Thy1 and N901 (NCAM, CD56). The function of the carbohydrate antigens is particularly uncertain in the light of differences in expression between corresponding human and mouse cells. Despite the lack of a clear function for the carbohydrate antigens, a considerable number of genes are devoted to encoding glycosyltransferases, and the expression of these antigens during embryogenesis and cell differentiation is very carefully controlled. It has been suggested that the core structures of these carbohydrates, rather than the terminal structures commonly recognized as epitopes by the various antibodies used in these studies, are the key molecules to affect cell behaviour (Fenderson et al. 1987). Although there are differences in expression of SSEA1, SSEA3 and SSEA4 between mouse and human, globoseries structures are a common feature of these cells in both species: although murine EC cells do not express SSEA3 and SSEA4 they do strongly express the Forsman antigen, another globoseries structure that is absent from human cells (Willison et al. 1982). Some carbohydrate antigens, however, have been shown to have a function. For example, the Lewis-X (Le^x) structure recognized as SSEA1 (Gooi et al. 1981) may be involved in compaction at the morula stage of mouse embryo development (Bird & Kimber, 1984; Fenderson et al. 1984). A role for the carbohydrate structures has also been postulated in axon guidance in the developing nervous system (Dodd & Jessell, 1986), and we previously proposed that the ability of cytomegalovirus to induce inappropriate expression of SSEA1 might provide a partial explanation for its ability to disrupt neural development in infected fetuses (Andrews et al. 1989).

Our present study highlights both similarities and differences between EC and ES cells, from humans, and between these cell types in humans and mice. EC cells

are caricatures of ES cells, adapted for tumour growth, and indeed many human EC cells do not differentiate significantly, presumably reflecting the selective advantage of accumulated mutations that interfere with differentiation (Andrews & Goodfellow, 1980; Duran et al. 2001). Nevertheless, the similarities of differentiation demonstrated by surface antigen changes between human EC cells that do differentiate, like NTERA-2, and hES cells is notable. The patterns induced by RA and HMBA are comparable, even if DMSO does not seem to be an effective inducer of the EC cells. It is also notable that neuroectoderm differentiation is a common feature of EC and hES differentiation, even though the hES cells evidently generate a greater diversity of cell types. Therefore, while it is clear that studies of hES cells can provide insights into the process of development in a way pertinent to human embryogenesis, human EC cells can in some circumstances provide convenient surrogates, lessons from which can be applied to understanding ES cell biology.

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EXHIBIT 5

Differentiation of Human Embryonic Stem Cells to Cardiomyocytes

Role of Coculture With Visceral Endoderm-Like Cells

Christine Mummery, PhD; Dorien Ward-van Oostwaard; Pieter Doevendans, MD, PhD; Rene Spijker; Stieneke van den Brink; Rutger Hassink, MD; Marcel van der Heyden, PhD; Tobias Opthof, PhD; Martin Pera, PhD; Aart Brutel de la Riviere, MD, PhD; Robert Passier, PhD; Leon Tertoolen, PhD

Background—Cardiomyocytes derived from human embryonic stem (hES) cells could be useful in restoring heart function after myocardial infarction or in heart failure. Here, we induced cardiomyocyte differentiation of hES cells by a novel method and compared their electrophysiological properties and coupling with those of primary human fetal cardiomyocytes.

Methods and Results—hES cells were cocultured with visceral-endoderm (VE)–like cells from the mouse. This initiated differentiation to beating muscle. Sarcomeric marker proteins, chronotropic responses, and ion channel expression and function were typical of cardiomyocytes. Electrophysiology demonstrated that most cells resembled human fetal ventricular cells. Real-time intracellular calcium measurements, Lucifer yellow injection, and connexin 43 expression demonstrated that fetal and hES-derived cardiomyocytes are coupled by gap junctions in culture. Inhibition of electrical responses by verapamil demonstrated the presence of functional α_{1c}-calcium ion channels.

Conclusions—This is the first demonstration of induction of cardiomyocyte differentiation in hES cells that do not undergo spontaneous cardiogenesis. It provides a model for the study of human cardiomyocytes in culture and could be a step forward in the development of cardiomyocyte transplantation therapies. **(*Circulation*. 2002;107:2733-2740.)**

Key Words: electrophysiology ■ myocytes ■ stem cells

Ischemic heart disease is the leading cause of mortality in the western world. Oxygen deprivation and subsequent reperfusion initiate irreversible cell damage, eventually leading to cell death and loss of function. Strategies to regenerate damaged cardiac tissue by cardiomyocyte transplantation may limit postinfarction cardiac failure. We have shown previously that visceral-endoderm (VE)–like cell lines induce mouse P19 embryonal carcinoma (EC) and mouse embryonic stem (ES) cells to aggregate spontaneously in coculture and differentiate to cultures containing beating muscle.[1,3] This induction potential was specific for VE-like cells and was also observed when aggregates of P19EC cells were grown in conditioned medium from one VE-like cell line, END-2. Moreover, Dyer et al[4] have shown that END-2 cells can induce the differentiation of epiblast cells from the mouse embryo to undergo hematopoiesis and vasculogenesis and respecify prospective neuroectodermal cell fate. These effects were largely attributable to Indian hedgehog secreted by END-2 cells and VE of the mouse embryo.

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Molecular pathways leading to specification and terminal differentiation of cardiomyocytes from embryonic mesoderm during development are still unclear. Data derived from chick and amphibian suggested that cardiac progenitors require interaction with anterior endoderm and possibly the organizer for myocardial differentiation to take place.[5–7] More recently, primitive streak and visceral embryonic endoderm were shown to be important for the multistep induction through which cardiac progenitor cells acquired the competence to complete terminal differentiation at day 7.5 of gestation in mice.[8]

Here, we demonstrate that coculture of pluripotent human ES (hES) cell lines with END-2 cells induces extensive differentiation to 2 distinctive cell types from different lineages. One is epithelial; it forms large cystic structures that stain positively for α-fetoprotein and is presumably extraembryonic VE; the others are grouped in areas of high local density and beat spontaneously. We show that these beating

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From the Hubrecht Laboratory (C.M., D.W.v.O., R.S., S.v.d.B., R.H., R.P., L.T.); the Departments of Medical Physiology (M.v.d.H., T.O.), Cardiothoracic Surgery (R.H., A.B.d.l.R.), and Cardiology (P.D.), University Medical Center Utrecht; and the Interuniversity Cardiology Institute of the Netherlands (C.M., P.D., R.P.), Utrecht, Netherlands; and the Monash Institute of Reproduction and Development, Melbourne Australia (M.P.).

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Correspondence to Christine Mummery, Hubrecht Laboratory, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands. E-mail christin@niob.knaw.nl

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cells are cardiomyocytes. Although differentiation of hES cells to cardiomyocytes has been described previously,[9-11] the hES cell lines used differentiate spontaneously to somatic derivatives in embryoid bodies, reminiscent of those formed by mES cells.[12] The present work is thus the first describing induction of cardiomyocyte differentiation in hES cells, which do not undergo cardiogenesis spontaneously, even at high local cell densities, and is the first direct electrophysiological comparison of hES-derived cardiomyocytes with primary human fetal cardiomyocytes in culture.

Methods

Cell Culture

END-2 cells and hES2 cells were cultured as described previously.[1,13] To initiate cocultures, END-2 cell cultures treated for 3 hours with mitomycin C (mit.C; 10 μg/mL)[1] replaced mouse embryonic fibroblasts (MEFs) as feeders for hES cells. Cocultures were then grown for up to 6 weeks and scored for the presence of areas of beating muscle from 5 days onward. HepG2 cells, a carcinoma cell line resembling liver parenchymal cells,[14] were cultured in DMEM plus 10% FCS. Cocultures were initiated as for END-2 cells. For electrophysiology, beating aggregates were dissociated by use of collagenase and replated on gelatin-coated coverslips.

Immunohistochemistry

Cells were fixed with 3.0% paraformaldehyde, then permeabilized with 0.1% Triton X-100. Undifferentiated hES colonies were stained overnight at 4°C with anti-oct4 (Sigma) and visualized by use of the avidin-biotin complex/horseradish peroxidase kit (DAKO) and the Fast 3,3'-diaminobenzidine tablet set (Sigma). For immunofluorescence antibodies against α-actinin, tropomyosin, and pan-cadherin (Sigma), myosin light chain (MLC)-2a and -2v (from Dr K. Chien, San Diego Institute of Molecular Medicine, University of California, San Diego School of Medicine, La Jolla, Calif), α_{1c} and $Ca_v1.2a$ (Alomone Laboratories), connexin 43 (Cx43) (Transduction Laboratories), and phalloidin-Cy3 (Sigma) were used in combination with fluorescence-conjugated secondary antibodies (Jackson Laboratories). Confocal images (Leica Systems) were made (63× objective) from 2D projected Z series.

Primary Human Adult and Fetal Cardiomyocytes

Primary tissue was obtained during cardiac surgery or after abortion after individual permission had been obtained by use of standard informed consent procedures and approval of the ethics committee of the University Medical Center, Utrecht. Adult cardiomyocytes were isolated and cultured as reported previously.[3] Fetal cardiomyocytes were isolated from fetal hearts (16 to 17 weeks) perfused by Langendorff's method and cultured on glass coverslips. For patch-clamp electrophysiology, cells were collected in Tyrode's buffer with low Ca^{2+}.[15]

Reverse Transcription–Polymerase Chain Reaction

RNA was isolated by use of Ultraspec (Biotecx Laboratories) and reverse transcribed (RT; 500 ng total RNA) as described previously.[16] Primer sequences and conditions for polymerase chain reaction (PCR) are given in Table 1. Products were analyzed on ethidium bromide–stained 1.5% agarose gel. β-Actin or β-tubulin was used as RNA input control.

Electrophysiology

Data were recorded from cells at 33°C in spontaneously beating areas by use of an Axopatch 200B amplifier (Axon Instruments Inc). Cell-attached patches were made in the whole-cell voltage-clamp mode. The pipette offset, series resistance, and transient cancellation were compensated; subsequent action potentials were recorded by switching to the current-clamp mode of the 200B amplifier. Output signals were digitized at 4 kHz by use of a Pentium III equipped with an AD/DAC LAB PC+ acquisition board (National Instruments). Patch pipettes with a resistance between 1 and 3 MΩ were used. Bath medium was (mmol/L) 140 NaCl, 5 KCl, 2 $CaCl_2$, and 10 HEPES, adjusted to pH 7.45 with NaOH. Pipette composition (mmol/L) was 145 KCl, 5 NaCl, 2 $CaCl_2$, 4 EGTA, 2 $MgCl_2$, and 10 HEPES, adjusted to pH 7.30 with KOH. Verapamil was used at 5 μmol/L.

Calcium Measurements

Cells were labeled for 15 minutes at 37°C with 10 μmol/L fura 2-AM. The light from 2 excitation monochromators (SPEX fluorolog, SPEX Industries) was rapidly alternated between 340 (slit width: 8) and 380 (slit width: 8) nm and coupled into a microscope

TABLE 1. PCR Primers and PCR Conditions

Gene/Primer	Product Size, bp	Annealing Temperature, °C
Oct-4		
5′-GAGAACAATGAGAACCTTCAGGAGA	215	55
5′-TTCTGGCGCCGGTTACAGAACCA		
α-Actinin		
5′-GGCGTGCAGTACAACTACGTG	580	56
5′-AGTCAATGAGGTCAGGCCGGT		
ANF		
5′-GAACCAGAGGGGAGAGACAGAG	406	61
5′-CCCTCAGCTTGCTTTTTAGGAG		
MLC-2v		
First round		
5′-GCGCCAACTCCAACGTGTTCT		55
5′-GTGATGATGTGCACCAGGTTC		
Nested PCR		
5′-AGGAGGCCTTCACTATCATGG	444	55
5′-GTGATGATGTGCACCAGGTTC		
MLC-2a		
5′-GAGGAGAATGGCCAGCAGGAA	449	55
5′-GCGAACATCTGCTCCACCTCA		
Phospholamban		
5′-ACAGCTGCCAAGGCTACCTA	191	55
5′-GCTTTTGACGTGCTTGTTGA		
α_{1c}		
5′-CTGGACAAGAACCAGCGACAGTGCG	562	56
5′-ATCACGATCAGGAGGGCCACATAGGG		
Kv4.3		
5′-CTGGACAAGAACCAGCGACAGTGCG	322	55
5′-ATCACGATCAGGAGGGCCACATAGGG		
KvLQT1		
5′-TTCTTGGCTCGGGGTTTGCC	723	58
5′-TGTTGCTGCCGCGATCCTTG		
β-Actin		
5′-CCTGAACCCTAAGGCCAACCG	400	55
5′-GCTCATAGCTCTTCTCCAGGG		
β-Tubulin		
5′-TGGCTTTGCCCCTCTCACCA	369	61
5′-CGGCGGAACATGGCAGTGAA		

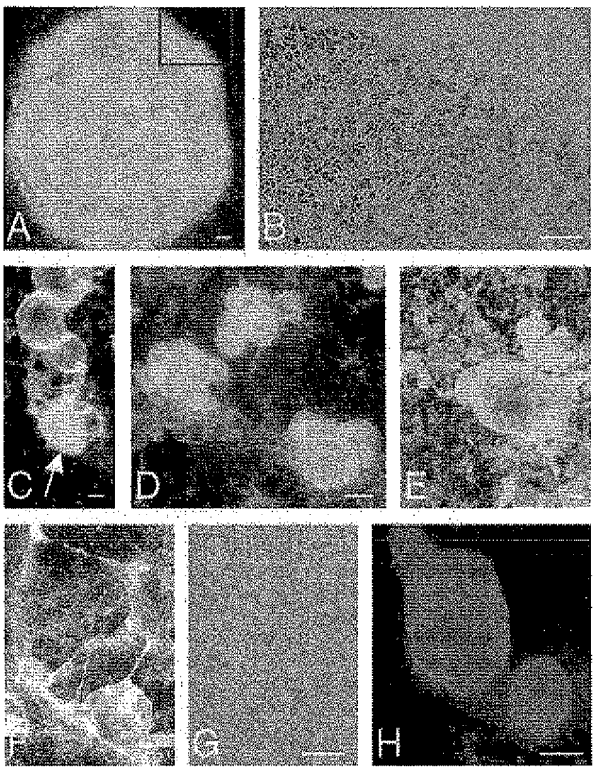

Figure 1. Induction of differentiation of hES cells by coculture with VE-like mEND-2 cells. A, Undifferentiated hES cell colony on MEF "feeder cells." B, Nuclear oct-4 staining of undifferentiated cells in area of colony indicated in A. C, hES cells after 11 days of coculture with END-2 cells with beating aggregate (arrow). D, Various morphologies of beating muscle aggregates. E, Phase-contrast image showing beating muscle areas in hES/END-2 coculture. F, Phase-contrast image of a nonbeating area with cardiomyocyte morphology. G, Dissociated hES aggregate, replated and beating as used for electrophysiology. H, Cystic structures stained with α-fetoprotein. Bar=100 μm.

via a UV-optic fiber. Fluorescence intensity images were recorded from living cells at a maximal rate of 120 ms/pair and corrected for background fluorescence. Calibration used the minimal ratio (R_{min}) after addition of 5 μg/mL ionomycin and 4 mmol/L EGTA (pH 8) to the cells and the maximal ratio (R_{max}) after addition of 5 μg/mL ionomycin and 10 mmol/L $CaCl_2$. The calcium concentration was calculated as follows: $(R-R_{min})/(R_{max}-R)\times sf2/sb2\times K_d$, where sf2 indicates the free dye concentration at 380 nm at saturating calcium conditions and sb2 is the calcium-bound dye concentration at 380 nm at saturating calcium conditions.[17]

Dye Coupling

A filtered solution of 3% wt/vol Lucifer yellow lithium salt (Molecular Probes) in 150 mmol/L LiCl was microinjected through Quick-fill glass microelectrodes (Clark Electromedical Instruments). Dye was injected into one of a group of spontaneously beating cells by a 1-Hz square pulse (50% duty cycle), amplitude of 5×10^{-9} A. Directly after injection, confocal laser scanning microscope images were made of the injected areas.

Results

Cardiomyocyte Differentiation of hES Cells

Of the hES cells maintained by coculture with mit.C-treated MEFs in FCS-containing medium[3] (Figure 1A), ≈60% showed nuclear staining for oct-4; flattened cells were negative (Figure 1B). Oct-4 expression thus correlated with phenotypic characteristics of undifferentiated cells. hES cells were subcultured by transferring small clumps of undifferentiated cells onto either new MEFs or END-2 cells. After ≈5 days, epithelial cells appeared, which gradually become fluid-filled cysts (Figure 1C). These stained for α-fetoprotein (Figure 1H), suggesting that they represent extraembryonic VE. By 10 days, areas of rhythmically contracting cells in more solid aggregates became evident in the hES–END-2 cocultures (Figure 1C, arrow) with a variety of overall morphologies (Figure 1D). In a 12-well plate, 35±10% of the wells (n=30) contained beating areas, each of which could be dissociated and replated to yield up to 12 new colonies of beating cells with a 2D rather than 3D morphology (Figure 1G); this facilitated access to the cells for electrophysiology. Each beating area consisted of 10 to 200 cardiomyocytes. Control cultures on MEFs showed no evidence of beating muscle or extensive cyst formation but had formed very large colonies with many flattened cells at the edges (not shown). Conversely, hES on HepG2 cells did form areas of beating muscle, usually attached to HepG2 cell colonies.

Before and after dissociation, hES-derived cardiomyocytes beat 35 to 90 times per minute (Table 2). Cardiomyocyte

TABLE 2. Cardiomyocyte Action Potential Properties

	Upstroke, Volts/s	Repolarization 10%, mV	Repolarization 10%, ms	Repolarization 90%, mV	Repolarization 90%, ms	Overshoot, mV	Amplitude, mV	Resting Potential, mV	Frequency, Hz	n (Total)
Pacemaker hES cells, mean	2.6	4.8	72.0	−20.8	134	18.0	32.0	−20.8	1.2	1 (33)
Atrium-like hES cells, mean±SEM	8.5±0.4	9.9±0.8	28.5±11.0	−38.7±0.6	121.8±3.8	26.0±1.0	60.8±3.2	−38.7±0.6	1.5±0.1	2 (33)
Atrium cells, fetal 16 weeks, mean±SEM	1.2±0.3	10.9±3.3	58.9±10.0	−34.9±10	164.9±14.3	16.6±3.8	57.2±5.0	−34.9±1.6	1.0±0.1	(8)
Ventricular-like hES cells, mean±SEM	7.0±0.8*	16.0±1.6	123.9±19.5	−48.0±1.7	436.4±55.3	24.0±1.9	80.0±3.5	−48.0±1.7	0.6±0.1	28 (33)
Ventricular cells, fetal 16 weeks, mean±SEM	8.9±4.3†	16.7±2.7	80.2±13.2	−38.5±1.6	370.0±45.8	23.6±3.5	69.0±9.1	−38.5±1.6	0.8±0.1	(6)

*Measured maximum: 20.0 (V/s).
†Measured maximum: 26.7 (V/s).

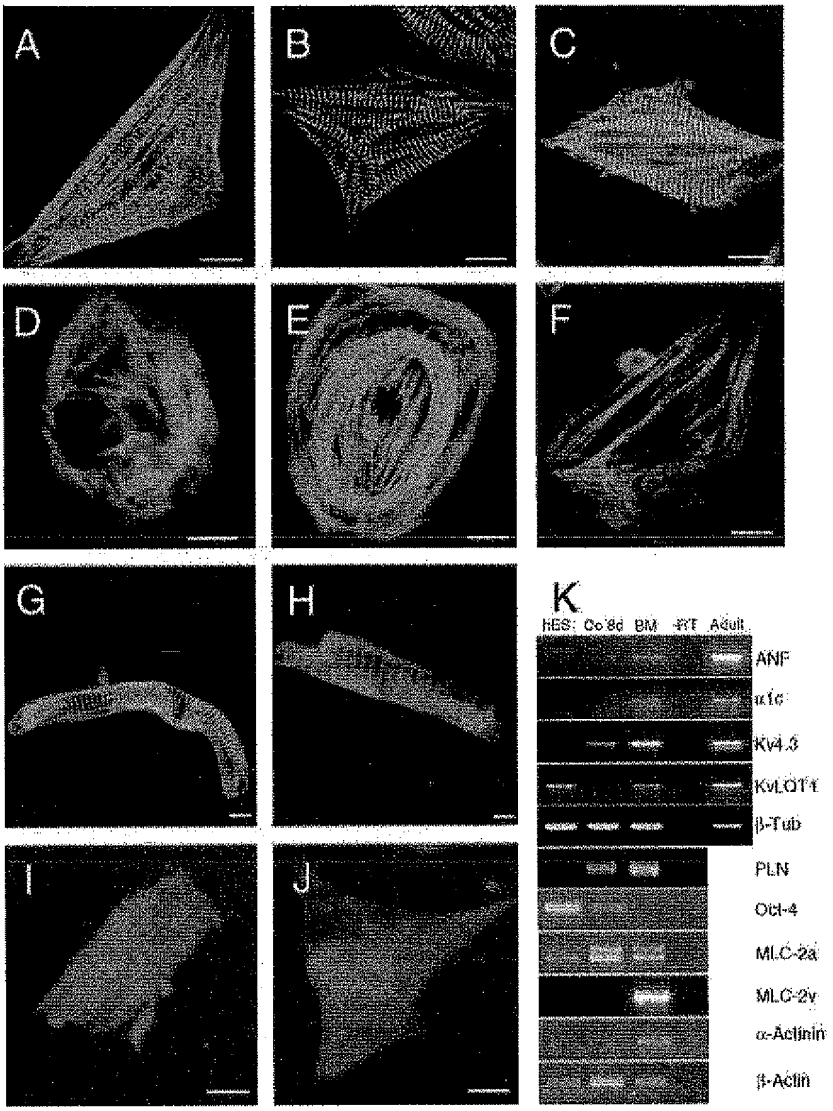

Figure 2. Cardiomyocyte markers and ion channels in hES cells and primary human fetal and adult cardiomyocytes. A, D, and I, hES-derived cardiomyocytes. Human fetal ventricular (B, E, J) and atrial (C, F) cardiomyocytes. Adult human ventricular (G) and atrial (H) cardiomyocytes. Cells were stained with anti–α-actinin (green) (A–C, G, H), anti–MLC-2a (red) (C, H), anti–MLC-2v (red) (G), anti-tropomyosin (green) (D–F), and for viable ryanodine receptors (I, J). K, RT-PCR on hES cells, hES cells cocultured for 8 days with END-2 cells (Co 8d), hES beating muscle (BM), adult human heart, and directly on RNA (-RT).

colonies could be frozen and sometimes resumed beating on thawing. To characterize the cardiomyocytes further, we carried out immunofluorescent staining for sarcomeric proteins, used BIDOPY-ryanodine as a vital stain for ryanodine receptors in the sarcoplasmic reticulum, and analyzed the expression of ion channels by RT-PCR. In each case, we used primary human fetal and adult atrial and ventricular tissue as controls. The data showed that hES-derived cardiomyocytes exhibited sarcomeric striations when stained with α-actinin (Figure 2A), organized in separated bundles. These were reminiscent of the bundles observed in human fetal cardiomyocytes (Figure 2, B and C), although the individual sarcomeres were less well defined. The morphology was different from the highly organized, parallel bundles in cells from biopsies of adult human heart (Figure 2, G and H). hES-derived cardiomyocytes also stained with MLC-2a, MLC-2v (not shown), and tropomyosin (Figure 2D); again, the sarcomeres were less evident than in human fetal and adult cardiomyocytes (Figure 2, E, F, and H).

Expression of Cardiac Ion Channels and Stem Cell/Sarcomere Markers in hES/END-2 Cocultures

Expression of cardiac-specific ion channels was determined in undifferentiated hES cells and at 8 and 15 days after initiation of coculture with END-2 cells (Figure 2K). As shown previously by others,[10] areas of beating hES-derived cardiomyocytes express atrial natriuretic factor. Expression of the α-subunits of the cardiac-specific L-type calcium channel (α_{1c}) and the transient outward potassium channel (Kv4.3) was also detected, the expression of Kv4.3 preceding onset of beating by several days. RNA for the delayed rectifier potassium channel KvLQT1 was found in undifferentiated cells, but transcripts disappeared during early differentiation and reappeared later.

Over a similar time course, expression of *oct-4* was reduced, whereas transcripts for α-actinin, MLC-2a, and MLC-2v became detectable (Figure 2K), reflecting the results of antibody staining.

Electrophysiology

Patch-clamp electrophysiology on dissociated hES cardiomyocytes showed that different electrical phenotypes were

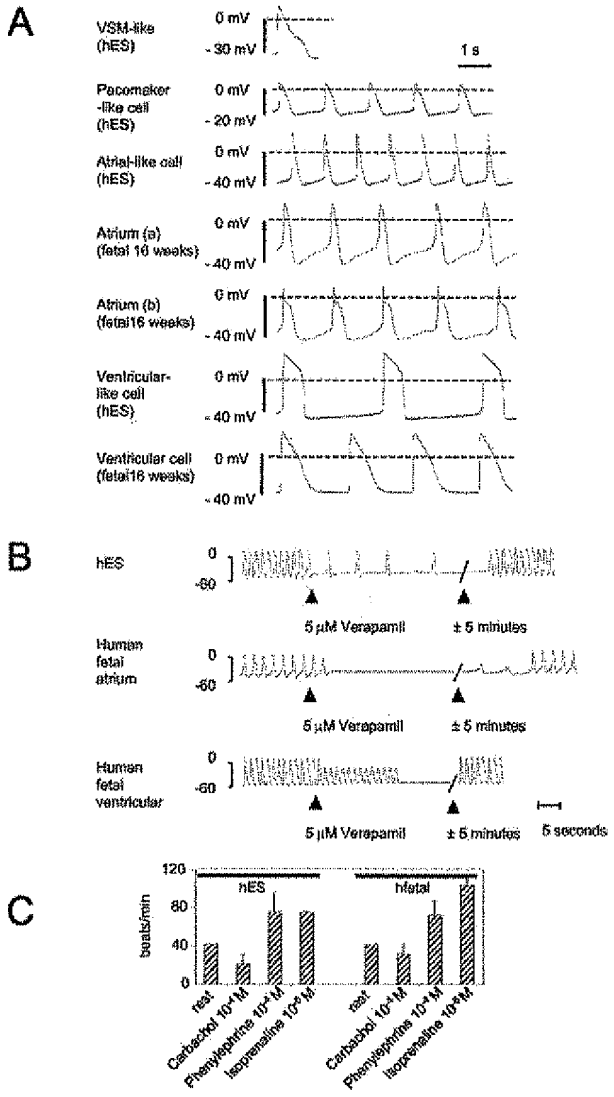

Figure 3. Action potentials and chronotropic responses. A, Action potentials in hES-derived beating cardiomyocytes and isolated human fetal ventricular and atrial cells. B, Effect of verapamil on action potentials in hES-derived and primary human fetal cardiomyocytes (hfetal). C, Chronotropic responses of hES and human fetal cardiomyocytes to different stimuli; mean beat frequency± SEM

present (Figure 3A). Ventricular-like action potentials predominated (28 of 33; Table 2), but atrial-like (n=2), pacemaker-like (n=1), and vascular smooth muscle–like cells (n=2) were also found. In areas in which the cells were not beating but had adopted morphologies indistinguishable from those of beating areas (Figure 1F), current injection was sufficient to induce repeated action potentials and sustained synchronous rhythmic contractions. Transcripts for MLC-2v were also detected by RT-PCR in nonbeating, myocyte-like areas (not shown); scoring beating muscle may thus underestimate the number of cardiomyocytes present in culture. The upstroke velocities (Volts/s) for the ventricular-like cells were low (8 Volts/s) but comparable to those in cultured human fetal ventricular cardiomyocytes, although incidental peak values were found (Table 2). α_1-Adrenoceptors, β_1-adrenoceptors (regulated via a cAMP-dependent mechanism), and nicotinic acetylcholine receptors are known to influence cardiac function. Chronotropic responses of dissociated hES cardiomyocytes were also compared with human fetal ventricular cells (Figure 3C). Addition of carbachol decreased the beating rate of hES-derived cardiomyocytes and human fetal ventricular cells, whereas phenylephrine and isoprenaline increased the rate in both cell types. Similar effects were reported in mES-derived cardiomyocytes[18] and mouse fetal cells.[19]

$[Ca^{2+}]_i$ Transients in Differentiated hES Cells

Calcium oscillations were recorded in dissociated groups of spontaneously beating hES cardiomyocytes (Figure 4). The continuous character of the repetitive line scans in Figure 4B in the left-to-right direction, compared with the vertical lines in 4C, shows that the action potential in Figure 3A propagates in a top-down direction and indicated tightly developed cell-to-cell coupling in this synchronously contracting group of cells. Regular repetitive oscillations in $[Ca^{2+}]_i$ are found in single hES cardiomyocytes (Figure 4E). Coupling between cells was confirmed by Lucifer yellow injection into single cells; the dye spread within minutes to other cells within the group in both hES-derived (Figure 5E) and primary fetal cardiomyocytes (not shown). Cx43 staining (Figure 5, B and D) indicated the presence of gap junctions. Staining with a pan-cadherin antibody also indicated the presence of adherens junctions between cells in fetal and hES-derived cardiomyocytes (Figure 5, A and C).

L-type calcium channels compose the predominant route for calcium entry into cardiac myocytes and are key components in excitation-contraction coupling. A specific α_{1C} antibody stained cardiomyocytes in both differentiated hES cultures (Figure 4F) and human fetal ventricular cells (Figure 4G), in agreement with the RT-PCR data (Figure 2K).

Discussion

Before hES cells can be applied clinically, it is important to control their growth and differentiation. Both embryonic and adult stem cells from the mouse apparently respond to cues within the mouse embryo to differentiate to (virtually) all somatic tissues (reviewed by Passier and Mummery[20]). If these cues and the signal transduction pathways they activate could be identified, this knowledge could be used to control differentiation of stem cells in culture and in vivo. Here, we have identified visceral endoderm as a cellular source of signals that result in human ES cells differentiating to cardiomyocytes with characteristics of fetal ventricular, atrial, or pacemaker cells. This is the first time that inductive cellular sources of signals have been identified that result in human ES cells forming cardiomyocytes, although various studies have shown that cells with endoderm-like properties have this effect on mouse ES and EC cells.[1,3,21–23] VE (END-2) and liver parenchymal (HepG2) cells share similar protein secretion profiles, so their ability to induce comparable responses in ES cells is not surprising. In contrast to mouse ES cells, in our hands, human ES cells do not easily form embryoid bodies when grown as aggregates and never show "spontaneous"

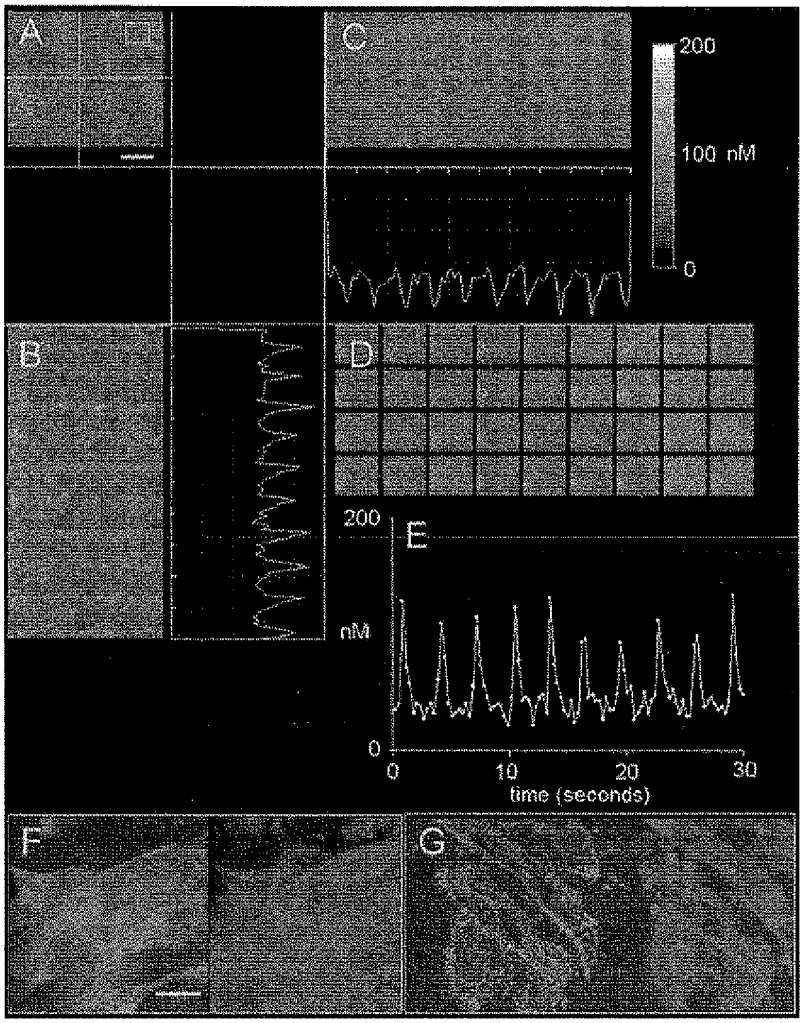

Figure 4. Calcium transients and L-type calcium channels. A, First image of an image stack (100 images; total time, 30 seconds) of a group of 7 cells. Lines indicate line scans in time through image stack. B, Intensity plot of horizontal line through image stack in time (time running from top to bottom). C, Intensity plot of vertical line from image (time running from left to right). D, Image stamps of first 36 images with time. E, Calcium transients in a single cell from upper right corner of image in A. Dots are average value of same region of interest in one cell in each slice in image stack. F and G, Confocal images of α-actinin–positive (green) and α1C (red)–positive cells in hES (F) and human fetal ventricular cardiomyocytes (G).

differentiation to cardiomyocytes, even at high cell densities in overgrowths. This contrasts with other reports[9,11] in which the hES cells do form embryoid bodies containing cardiomyocytes. Identification of a reproducible source of inductive signals nevertheless represents an important step forward, comparable to a recent report showing differentiation of hES cells to hematopoietic cells after coculture with bone marrow stromal cells or yolk sac endothelial cells. It has been suggested that signals from the endoderm, such as bone morphogenic proteins (BMPs), fibroblast growth factors, and repressors of wnt signaling, may be important for cardiac development.[24] Endoderm in the mouse embryo expresses *BMP2*[25] and inhibitors of wnt signaling.[26,27] Direct addition of BMP2 to hES cells, however, did not result in cardiomyocyte differentiation; on the contrary, they formed extraembryonic endoderm (data not shown). We therefore think it unlikely that activation of the BMP signaling pathway is the primary event initiated by END-2/hES cell coculture. Likewise, we saw no obvious effect of fibroblast growth factors. These signals could, however, be involved later in differentiation of nascent mesoderm to cardiomyoblasts. Late addition of the demethylating agent 5-azacytidine to developing embryoid bodies has also been shown to be more effective than early addition.[11] Careful stepwise analysis of hES cell differentiation and approaches recapitulating or mimicking endogenous signals in the embryo are the most likely to increase the efficiencies of hES differentiation to specific lineages. In addition, transplantation of committed but immature cells that have retained the capacity to form functional junctions with host cells are likely to have the least chance of introducing arrhythmias.

Staining for junctional proteins showed that the hES-derived cardiomyocytes were very immature, although real-time determination of intracellular Ca^{2+} concentrations clearly showed that the cells were electrically coupled. Kehat et al[9] recently reported similar findings in independently derived hES-cardiomyocytes. It will be of interest to subject these hES-derived cardiomyocytes to oscillating stress to see whether the sarcomeric structure matures to the adult phenotype.

In the adult mammalian myocardium, cellular Ca^{2+} entry is regulated by the sympathetic nervous system. L-type Ca^{2+} channel currents are markedly increased by β-adrenergic agonists, which contribute to changes in rate and contractile activity of the heart. Exactly how this Ca^{2+}

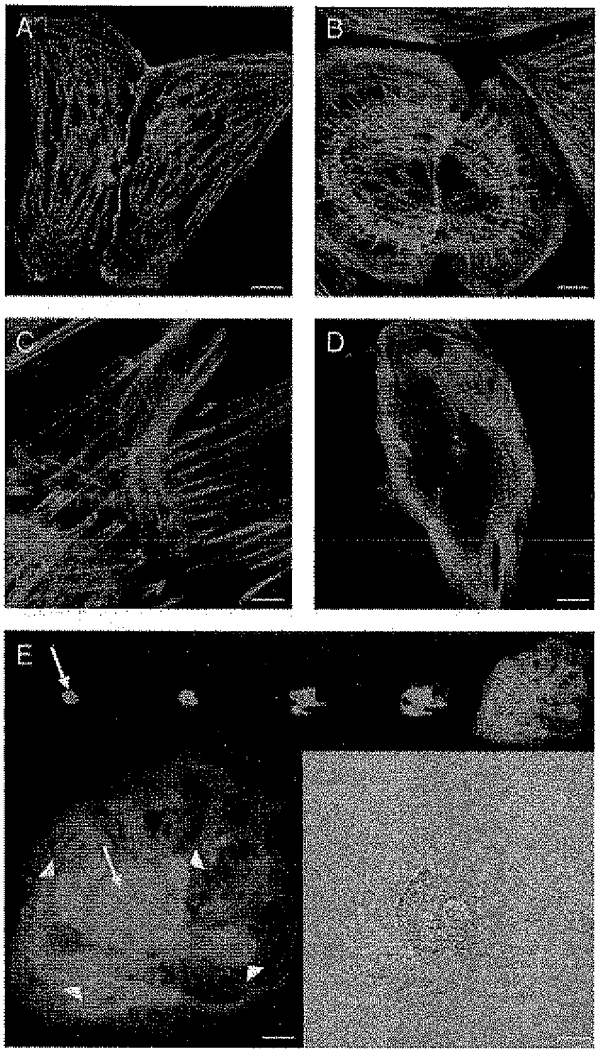

Figure 5. Junctional communication in hES-derived and human fetal cardiomyocytes. A and B, Human ventricular fetal cardiomyocytes. C and D, hES-derived cells. Double staining of phalloidin (red) and anti-pan-cadherin (green) (A and C) or anti-Cx43 (green) (B and D). E, Injection of Lucifer yellow into a single cell (arrows) within a group of beating hES-derived cardiomyocytes results in dye spreading to multiple cells (arrowheads, left bottom; phase contrast, right bottom) within minutes as determined by 2D projected *Z* series (top, left to right).

regulatory system is established in development is not yet clear. Our data indicate that the L-type Ca^{2+} channels in hES-derived cardiomyocytes and fetal cardiomyocytes responded to adrenergic stimuli, indicating a fully developed and connected downstream pathway. Verapamil, which specifically blocks L-type Ca^{2+} channels, inhibited action potentials in fetal and hES-derived cardiomyocytes, as expected. This contrasts with mouse fetal myocytes and mES-derived cardiomyocytes, in which early cells were nonresponsive despite the presence of L-type Ca^{2+} channels. Here, the lack of cAMP-dependent protein kinase appeared to be the limiting factor.[12,19] Thus, although hES-derived and early human fetal cardiomyocytes show some features in common with early mouse cardiomyocytes, their calcium channel modulation resembles that in the adult mouse. hES cells may thus represent an excellent system for studying changes in calcium channel function during early human development, which appears to differ significantly from that in mice. Furthermore, the appropriate calcium handling makes the cells more suitable for transplantation. Interesting was the observation of cells with plateau- and nonplateau-type action potentials in the fetal atrial cultures. These have been described dispersed throughout the atrium of intact fetal hearts[28] and have been considered a possible index of specialization of an atrial fiber, although their significance is not clear. The nonplateau type was not observed among the hES-derived cardiomyocytes.

Finally, vital fluorescent staining with ryanodine or antibodies against cell surface α_{1c} ion channels allowed cardiomyocytes to be identified in mixed cultures. This may provide a means of isolating cells for transplantation without genetic manipulation or compromising viability.

Acknowledgments

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EXHIBIT 6

Characterization and Enrichment of Cardiomyocytes Derived From Human Embryonic Stem Cells

Chunhui Xu, Shailaja Police, Namitha Rao, Melissa K. Carpenter

Abstract—Cell replacement therapy is a promising approach for the treatment of cardiac diseases, but is challenged by a limited supply of appropriate cells. We have investigated whether functional cardiomyocytes can be efficiently generated from human embryonic stem (hES) cells. Cardiomyocyte differentiation was evaluated using 3 parent (H1, H7, and H9) hES cell lines and 2 clonal (H9.1 and H9.2) hES cell lines. All cell lines examined differentiated into cardiomyocytes, even after long-term culture (50 passages or ≈260 population doublings). Upon differentiation, beating cells were observed after one week in differentiation conditions, increased in numbers with time, and could retain contractility for over 70 days. The beating cells expressed markers characteristic of cardiomyocytes, such as cardiac α-myosin heavy chain, cardiac troponin I and T, atrial natriuretic factor, and cardiac transcription factors GATA-4, Nkx2.5, and MEF-2. In addition, cardiomyocyte differentiation could be enhanced by treatment of cells with 5-aza-2′-deoxycytidine but not DMSO or retinoic acid. Furthermore, the differentiated cultures could be dissociated and enriched by Percoll density centrifugation to give a population containing 70% cardiomyocytes. The enriched population was proliferative and showed appropriate expression of cardiomyocyte markers. The extended replicative capacity of hES cells and the ability to differentiate and enrich for functional human cardiomyocytes warrant further development of these cells for clinical application in heart diseases. (*Circ Res.* 2002;91:501-508.)

Key Words: human embryonic stem cells ■ cardiomyocytes ■ differentiation ■ pharmacological responses ■ cell separation

Human cardiomyocytes proliferate and mature during gestation; however, these cells terminally differentiate soon after birth.[1] It is thus generally accepted that cardiomyocytes cannot be regenerated once heart tissue is damaged by trauma such as ischemic conditions leading to cardiac infarction.[1,2] Although it appears that somatic stem cells can migrate to heart tissue and differentiate into cardiomyocytes,[3,4] such events may not be sufficient to reverse the pathological conditions. To enhance the biological function of the damaged heart, cell transplantation may be an effective therapy. Animal studies have used various types of cells for transplantation, including fetal and neonatal cardiomyocytes, skeletal and smooth muscle, fibroblasts, and bone marrow–derived cells.[4–11] Many cell types including fetal and neonatal cardiomyocytes appear to be promising candidates because of their ability to integrate into the host tissue[7,12,13] and to improve heart function.[14,15] Although this type of transplantation is promising, the source of cells such as human fetal and neonatal cardiomyocytes for cell therapies is, however, limited. This issue is particularly relevant because a significant percentage of transplanted fetal rat cardiomyocytes die posttransplantation.[16] It may therefore require either transplantation of large numbers of cardiomyocytes to achieve survival of adequate cell numbers or improvement of survival of transplanted cells.

Cardiomyocytes have been successfully derived from mouse embryonic stem (mES) cells and shown to form stable grafts in the mouse heart.[17–23] The availability of human embryonic stem (hES) cells[24,25] offers a possible solution to the poor availability of human cardiomyocytes for transplantation. hES cells have been successfully maintained in vitro for over 250 population doublings and retain stable phenotype and karyotype.[26,27] Furthermore, we have established a feeder-free system for culturing hES cells that maintains the potential of these cells to differentiate into cells of all 3 germ lineages, including beating cardiomyocytes.[27] This culture system will facilitate generation of large quantities of cells for therapeutic applications.

In the present study, we report that cardiomyocytes can be efficiently derived from hES cells using appropriate culture conditions. The cells express cardiac genes and respond appropriately to cardioactive drugs. hES cell–derived cardiomyocytes can be enriched by density separation and appear to retain appropriate phenotype, which will facilitate their use in cell replacement therapy.

Materials and Methods

Induction of Cardiomyocyte Differentiation

hES cells were maintained as described in the expanded Materials and Methods section (which can be found in the online data

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From the Geron Corporation, Menlo Park, Calif.
Correspondence to Chunhui Xu, PhD, Geron Corporation, 230 Constitution Dr, Menlo Park, CA 94025. E-mail cxu@geron.com

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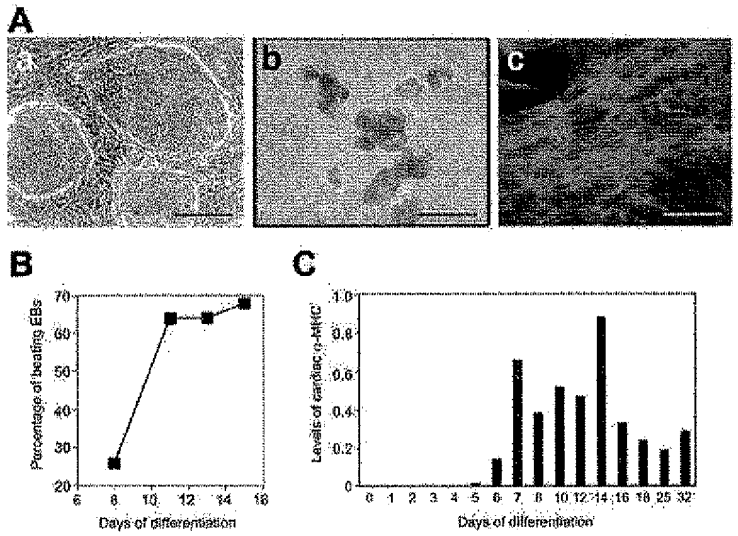

Figure 1. Differentiation of cardiomyocytes from hES cells. A, Confluent cultures of undifferentiated hES cells (a) were dissociated and cultured in suspension to form embryoid bodies (EBs) (b). EBs were transferred to gelatin-coated plates after 4 days in suspension culture to allow further differentiation into a heterogeneous cells, including spontaneously contracting cardiomyocytes that were positive for cTnI (c). Bar=400 μm for a and b and 50 μm for c. B, Percentage of EBs derived from H9.2 cells (passage 29+26, cells were subcloned from H9 at passage 29 and maintained for additional 26 passages) containing contracting cells during differentiation. C, Real-time RT-PCR analysis of cardiac α-MHC during differentiation of H1 cells (passage 29) normalized to 18S RNA.

supplement available at http://www.circresaha.org) and induced to differentiate as described below. Cells were dissociated into clumps using 200 U/mL collagenase IV (Invitrogen) at 37°C for 5 to 10 minutes and cultured in suspension using low attachment plates (Corning Inc) to form embryoid bodies (EBs). The differentiation medium contained 80% KO-DMEM, 1 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, 1% nonessential amino acids stock, and 20% FBS (Hyclone). After 4 days in suspension, EBs were transferred onto gelatin or poly-L-lysine–coated plates at ≈1 to 3 EBs/cm^2 and cultured for additional days as described in Results. The cultures were then examined for the presence of beating cells and subjected to analysis of gene expression or pharmacological studies. The effect of the differentiation reagents dimethyl sulfoxide (DMSO), all-trans retinoic acid (RA), or 5-aza-2′-deoxycytidine (5-aza-dC), which are known to enhance cardiomyocyte differentiation in murine embryonic carcinoma (mEC) P19 cells, mES cells, or mesenchymal stem cells,[26–30] respectively, was assessed at different times during differentiation. Cultures were exposed to the reagent at the beginning of treatment and returned to basal medium without the reagent after the treatment. The number of days of differentiation includes the days in which the cells were maintained in suspension. For example, differentiation day 6 is after cells were maintained in suspension for 4 days, plated, and cultured for an additional 2 days after plating.

hES cell–derived cardiomyocytes were characterized by immunostaining and RT-PCR and evaluated in vitro for responses to pharmacological agents as described in the online data supplement.

Percoll Enrichment of Cardiomyocytes

Differentiated hES cells containing beating cells were dissociated, resuspended in differentiation medium, and loaded onto a discontinuous Percoll gradient. Percoll (Pharmacia) was diluted in a buffer containing 20 mmol/L HEPES and 150 mmol/L NaCl. The gradient consisted of a 40.5% Percoll layer over a layer of 58.5% Percoll. After centrifugation at 1500*g* for 30 minutes, cell layers were apparent. Cells at different layers were collected, washed, resuspended in the differentiation medium, and plated for immunostaining, or collected for real-time RT-PCR analysis. For immunocytochemical analysis, the fractionated cells were seeded into chamber slides, cultured for an additional few days and immunostained.

Methods for dissociation of cardiomyocytes, immunostaining and RT-PCR are provided in the online data supplement.

Results

Cardiac differentiation was initiated by inducing EB formation from undifferentiated hES cells (Figure 1A). In order to monitor the presence of beating cells in individual EBs, EBs were seeded at low density after 4 days in suspension culture, and the locations of EBs in each well were recorded. The EBs attached and continued to proliferate and differentiate into a heterogeneous population of cells including beating cardiomyocytes. Spontaneously contracting cells appeared as clusters and were identified in approximately 25% of the individual EBs at differentiation day 8 and increased to as many as 70% of the EBs by day 16 (Figure 1B). The percentage of beating EBs usually increased over time until day ≈20 and maintained at this level. In some cases, the number of beating EBs declined due to the overgrowth of other cells, which sometimes caused the peeling of cells from the plate. We found that this problem can be overcome by lowering the EB seeding density, more gently aspirating during medium exchanges, or dissociating the cells and then replating them. In our hands, contracting cells could be found in long-term cultures maintained up to differentiation day 70.

Cardiomyocyte formation in EB cultures was seen in 3 hES cell lines as well as 2 clonal lines tested (H1, H7, H9, H9.1, and H9.2). hES cells maintained for 50 passages (≈260 population doublings) retained the capacity to differentiate into cardiomyocytes (see an example in Figure 1B).

Expression of Cardiac Markers in hES-Derived Cardiomyocytes

hES cell–derived cardiomyocytes express cardiac-specific troponin I (cTnI), a subunit of the troponin complex that provides a calcium-sensitive molecular switch for the regulation of striated muscle contraction.[31] We found that cTnI was detected only in the beating regions of the culture. A representative cTnI-positive area is shown in Figure 1Ac. The presence of cTnI in the contracting cells was also confirmed by Western blot, which showed that cTnI was expressed in differentiated hES cultures containing contracting cells, but not in undifferentiated hES cells or differentiated cultures with no evidence of contracting cells (data not shown). Similar results were found in all cell lines tested.

Real-time RT-PCR assays showed that cardiac-specific α-MHC transcripts were undetectable in undifferentiated hES cell cultures or differentiated cultures at early stages, and

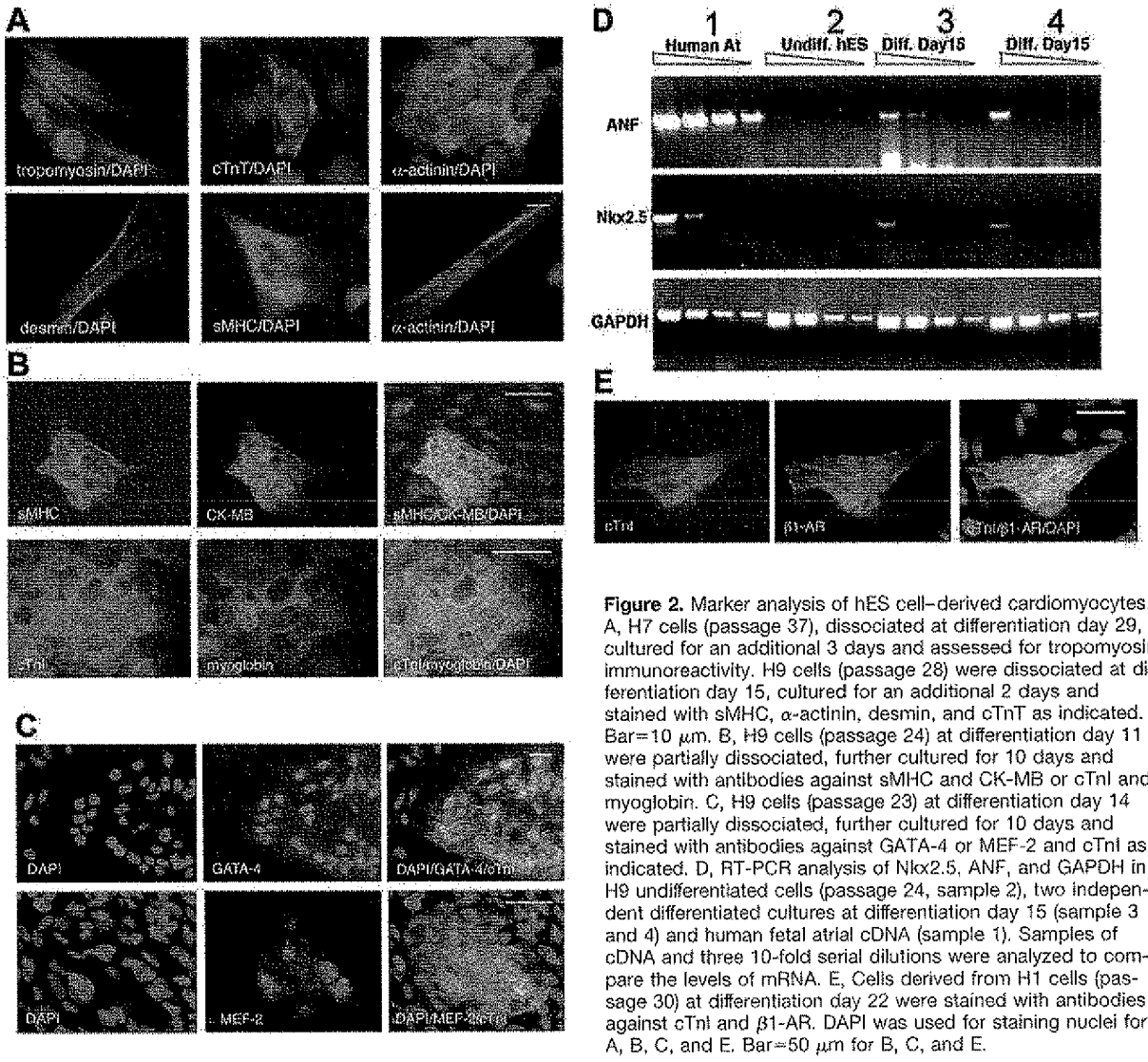

Figure 2. Marker analysis of hES cell–derived cardiomyocytes. A, H7 cells (passage 37), dissociated at differentiation day 29, cultured for an additional 3 days and assessed for tropomyosin immunoreactivity. H9 cells (passage 28) were dissociated at differentiation day 15, cultured for an additional 2 days and stained with sMHC, α-actinin, desmin, and cTnT as indicated. Bar=10 μm. B, H9 cells (passage 24) at differentiation day 11 were partially dissociated, further cultured for 10 days and stained with antibodies against sMHC and CK-MB or cTnI and myoglobin. C, H9 cells (passage 23) at differentiation day 14 were partially dissociated, further cultured for 10 days and stained with antibodies against GATA-4 or MEF-2 and cTnI as indicated. D, RT-PCR analysis of Nkx2.5, ANF, and GAPDH in H9 undifferentiated cells (passage 24, sample 2), two independent differentiated cultures at differentiation day 15 (sample 3 and 4) and human fetal atrial cDNA (sample 1). Samples of cDNA and three 10-fold serial dilutions were analyzed to compare the levels of mRNA. E, Cells derived from H1 cells (passage 30) at differentiation day 22 were stained with antibodies against cTnI and β1-AR. DAPI was used for staining nuclei for A, B, C, and E. Bar=50 μm for B, C, and E.

increased significantly after day 7 of differentiation (Figure 1C). In contrast, expression of hTERT, a gene expressed in undifferentiated hES cell cultures,[27,32] decreased during the process of differentiation (data not shown).

Other muscle markers were evaluated using dissociated hES cell–derived cardiomyocytes: sMHC, tropomyosin, α-actinin, desmin, and cardiac troponin T (cTnT) proteins were detected in single beating cells or clusters of cells (Figure 2A). Single stained cardiomyocytes showed spindle, round, and tri- or multiangular morphologies with striations characteristic of the sarcomeric structures of muscle cells. Immunostaining showed that 100% of sMHC-positive cells express cTnI, indicating that all the identified cells were cardiomyocytes. Furthermore, myogenin, a skeletal muscle–specific marker, was not detectable in the sMHC-positive cells by immunostaining, suggesting that the hES cell–derived cardiomyocytes were not expressing inappropriate proteins.

In addition to structural proteins, creatine kinase-MB (CK-MB) and myoglobin were also expressed by hES cell–derived cardiomyocytes (Figure 2B). CK-MB is found to be involved in high-energy phosphate transfer and facilitates diffusion of high-energy phosphate from mitochondria to myofibril in myocytes. Myoglobin is a cytosolic oxygen binding protein responsible for the storage and diffusion of oxygen within myocytes. Thus, hES cell–derived cardiomyocytes appear to have appropriate metabolic activity.

hES cell–derived cardiomyocytes also specifically expressed several cardiac transcription factors, including GATA-4, MEF-2, and Nkx2.5, in the differentiated cultures. These transcription factors are expressed in precardiac mesoderm and persist in the heart during development. GATA-4 immunoreactivity was found in nuclei of all cTnI-positive cells (Figure 2C). Western blots also indicated that GATA-4 is highly expressed in differentiated hES cells containing contracting cells but not in differentiated cultures that did not contain contracting cells (data not shown), indicating that GATA-4 is associated with cardiomyocyte differentiation. Similarly, MEF-2 was also expressed in nuclei of cTnI-positive cells as detected by immunostaining (Figure 2C).

Semiquantitative RT-PCR indicated that Nkx2.5 was expressed in hES cell–differentiated cultures containing beating cardiomyocytes, but undetectable in undifferentiated cultures (Figure 2D). Real-time RT-PCR analysis indicated that expression of Nkx2.5 is very low or nondetectable during H1 differentiation from day 0 to 6 and significantly increased at day 7 (data not shown). Therefore, hES cell–derived cardiomyocytes express cardiac transcription factors appropriately.

In addition, atrial natriuretic factor (ANF), a hormone that is actively expressed in both atrial and ventricular cardiomyocytes in developing heart, but is significantly downregulated in adult ventricular cells,[33] was found to be up-regulated during cardiac differentiation of hES cells as detected by a semiquantitative RT-PCR (Figure 2D).

Taken together, the above data indicate that hES cell–derived cardiomyocytes show characteristic gene expression patterns of developing cardiomyocytes.

Pharmacological Responses of hES Cell–Derived Cardiomyocytes

The in vitro function of hES cell–derived cardiomyocytes was examined by evaluating chronotropic effects of cardioactive drugs. Ion channels including L-type calcium channels play critical roles in cardiac contractile function.[34] RT-PCR analysis shows that α_1 subunit of L-type calcium channel is detected in differentiated cultures (data not shown). Therefore, we determined the effect of diltiazem, an ion channel blocker, on the beating frequency of hES cell–derived cardiomyocytes. Differentiated cells were incubated with various concentrations of the drug followed by measuring the beating frequency. Figure 3A shows that the beating frequency was decreased by diltiazem in a concentration-dependent manner; treatment with 10^{-7} mol/L diltiazem significantly reduced the frequency, and treatment with 10^{-5} mol/L stopped pulsatile contraction entirely. Contractions recovered to a normal rate 24 to 48 hours after removal of the drug. These results suggest functional ion channels exist in the hES cell–derived beating cardiomyocytes.

Cytosolic calcium is a crucial factor for controlling cardiomyocyte contraction and can be influenced by the interaction of adrenoceptors (ARs) with their ligands.[35] We therefore examined whether hES cell–derived cardiomyocytes expressed ARs by immunostaining with antibodies against AR and cTnI. The cardiomyocytes identified by cTnI expression were also immunoreactive for β_1-AR (Figure 2E) and α_1-AR (data not shown). To determine if ARs were functioning appropriately, contracting cells were treated with isoprenaline, a β_1-AR agonist, or phenylephrine, an α_1-AR agonist, and the rate of beating was monitored. As shown in Figures 3B and 3C, both isoprenaline and phenylephrine enhanced the contraction rate of hES cell–derived cardiomyocytes at differentiation day 15 to 20 in a dose-dependent manner. Unlike responses to isoprenaline or phenylephrine, cells at early stages (differentiation day 22 and 39) did not respond to clenbuterol, a β_2-AR agonist. However, cultures allowed to differentiate for a longer period of time (day 61 to 72) showed an increase in beating frequency (Figure 3E). These results suggest that differential responses of adrenoceptors occur

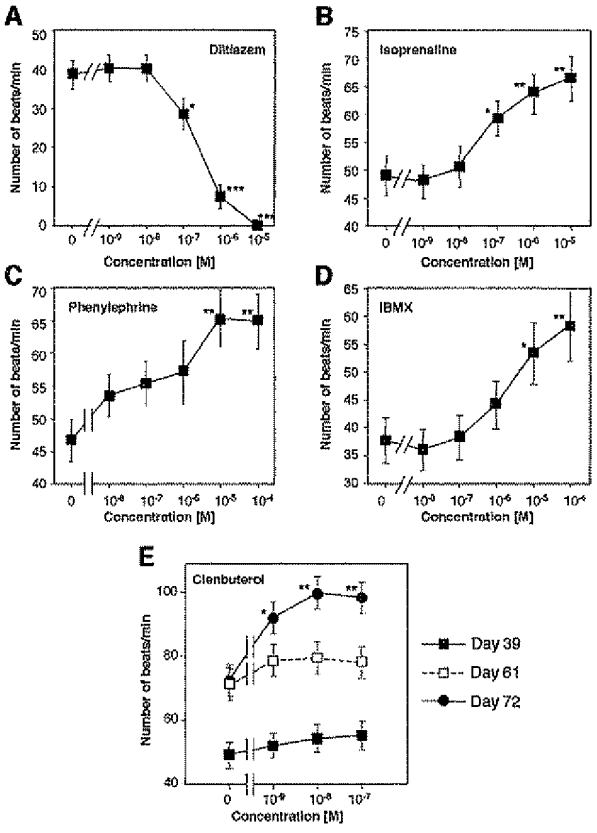

Figure 3. Studies of pharmacological responses. Effect of (A) diltiazem (a calcium channel blocker), (B) isoprenaline (a β_1-adrenoceptor agonist), (C) phenylephrine (an α1-adrenoceptor agonist), and (D) IBMX (an inhibitor of phosphodiesterases), on the contraction rate of cardiomyocytes derived from H9 cells (passage 31 to 32) or H7 cells (passage 49) at differentiation day 15 to 21. Effect of (E) clenbuterol (a β_2-adrenoceptor agonist) on H7 cells (passage 48) at differentiation day 39, 61, or 72. Each data point represents the mean±SEM pulsation rate. Statistical significance was tested by the ANOVA test: $^*P<0.05$, $^{**}P<0.005$, $^{***}P<0.0005$.

during cardiomyocyte differentiation from hES cells, similar to that seen with mES cell–derived cardiomyocytes.[36]

Application of isobutyl methylxanthine (IBMX), an inhibitor of phosphodiesterase (which converts cAMP into 5′AMP), resulted in a concentration-dependent increase of the contraction rate by IBMX (Figure 3D). These results indicate that the hES cell–derived cardiomyocytes respond appropriately to cardioactive drugs and this response may be mediated through a cAMP-dependent mechanism.[37]

Effect of Differentiation Induction Reagents on Cardiomyocyte Differentiation

In order to enhance cardiomyocyte differentiation, the effect of differentiation induction reagents was evaluated. DMSO and RA, which have been shown to induce cardiomyocyte differentiation in mEC P19 cells[28] and mES cells,[29] respectively, were evaluated but did not enhance hES cell cardiomyocyte differentiation (additional results in the online data supplement).

5-aza-dC has been shown to induce differentiation of mesenchymal stem cells presumably via demethylation of

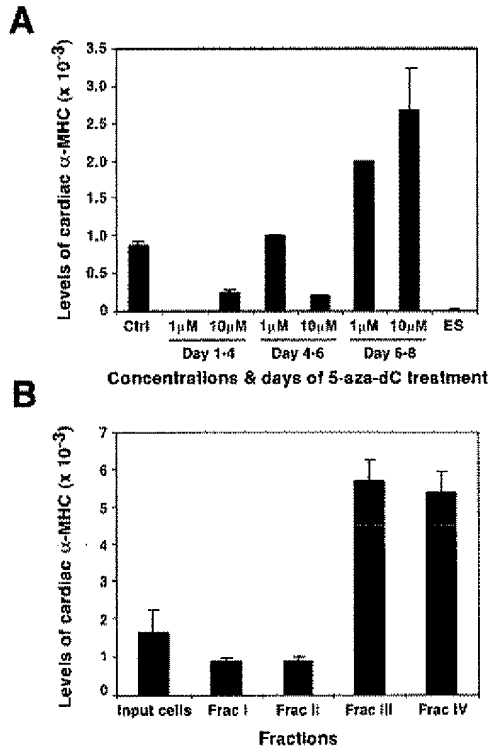

Figure 4. Enrichment of cardiomyocytes by 5-aza-dC treatment and Percoll separation. A, Effect of 5-aza-dC treatment on cardiac α-MHC mRNA levels of differentiation of H9 cells (passage 26). Cells were treated with 5-aza-dC at differentiation day 1 to 4, 4 to 6, or 6 to 8 and analyzed at differentiation day 15 for cardiac α-MHC mRNA levels by real-time RT-PCR Taqman analysis. Error bars that are not visible are smaller than the width of the symbol. ES indicates undifferentiated hES cells; Ctrl, untreated differentiated cell control. B, Effect of Percoll separation on enrichment of cardiomyocytes. H9 cells (passage 31) at differentiation day 22 were dissociated and separated by Percoll centrifugation. Cardiac α-MHC mRNA levels of cells in different fractions were compared with the starting material (input cells). 18S was used for normalization for A and B.

DNA.[30] To examine if 5-aza-dC affects cardiomyocyte differentiation of hES cells, hES cells were treated with 5-aza-dC at differentiation day 1 to 4, 4 to 6, or 6 to 8. Cells were harvested at day 15 and analyzed for cardiac α-MHC by real-time RT-PCR. Treatment of H9 or H1 cells with 5-aza-dC at day 6 to 8 significantly increased the expression of cardiac α-MHC (H9; data shown in Figure 4A). In contrast, a significant decrease in expression of cardiac α-MHC was observed when H9 or H1 cells were treated at differentiation day 1 to 4. In addition, the level of cardiac α-MHC decreased when H9 cells were treated with 10 μmol/L but not 1 μmol/L 5-aza-dC during differentiation day 4 to 6 compared with the nontreatment control. Immunostaining analysis of cTnI-positive cells indicated that the increase in α-MHC correlates with an increase in the number of cardiomyocytes (online data supplement). Therefore, 5-aza-dC appears to enhance cardiomyocyte differentiation from hES cells in a time-dependent manner. Further research is needed to characterize the complete phenotype of these cells.

Enrichment of Cardiomyocytes Using Discontinuous Percoll Gradients

In order to use hES cell–derived cardiomyocytes in therapeutic applications, it will be beneficial to produce a population of cells highly enriched for cardiomyocytes. We have used discontinuous Percoll gradients to successfully enrich hES cell–derived cardiomyocytes. An example is provided in online Table 2 (in the online data supplement available at http://www.circresaha.org) in which H7 cell–derived cardiomyocytes at differentiation day 21 were dissociated and applied to a discontinuous Percoll gradient (40.5% over 58.5%). After centrifugation, 2 layers of cells were observed: one on top of the Percoll (fraction I) and a layer of cells at the interface of the 2 layers of Percoll (fraction III). These 2 fractions, cells within the 40.5% Percoll layer (fraction II) and the 58.5% Percoll layer (fraction IV), and the starting material (input cells) were collected and cultured for 2 or 7 days before immunostaining. Although beating cells were observed in all fractions, fraction III and IV contained a higher percentage of beating cells. Quantitative analysis of triplicate wells showed that fraction III contained 36±3% sMHC-positive cells and fraction IV contained 70±5% sMHC-positive cells, whereas fraction I or II contained only 3% to 5% sMHC-positive cells 2 days after seeding (online Table 2). Compared with the starting material that contained 17±4% sMHC-positive cells, fraction IV showed a 4-fold enrichment. Similar results were observed for cells cultured for additional 7 days (online Table 2). We also applied the same separation procedure to H9 cells at differentiation day 22 and found that levels of cardiac α-MHC RNA in fractions III and IV were significantly higher than cells without the separation, confirming the enrichment (Figure 4B). Similar enrichment results (20% to 40% sMHC or cTnI-positive cells for fraction III and 50% to 70% sMHC or cTnI-positive cells for fraction IV) were observed in multiple experiments using H1 or H7 cells. These results indicate a significant enrichment of cardiomyocytes using a discontinuous Percoll gradient separation.

To characterize the Percoll-enriched cell populations, we performed immunostaining using antibodies against various markers. As shown in online Table 3, positive immunoreactivity for antibodies against cardiac α/βMHC, βMHC and sMHC was found in all cardiac cells as identified by cTnI-positive cells, but not in noncardiac cells. A representative image of cTnI and sMHC staining is shown in Figure 5. In addition, cTnI-positive cells expressed N-cadherin. Neither cardiac cells nor noncardiac cells expressed myogenin, AFP, or β-tubulin III, indicating the absence of skeletal muscle, endoderm cell types, or neurons in the Percoll-enriched culture. To examine if there were any undifferentiated hES cells in the population, surface markers for undifferentiated hES cells, SSEA-4 and Tra1-81, were analyzed. No detectable signal was found in either cardiac or noncardiac cells. Therefore, the Percoll-enriched cells did not appear to contain undifferentiated hES cells.

It has been reported that α-smooth muscle actin (SMA) is present in embryonic and fetal but not in adult cardiomyocytes.[38,39] Immunostaining results indicated that all cTnI-positive cells and a subset of cTnI-negative cells expressed

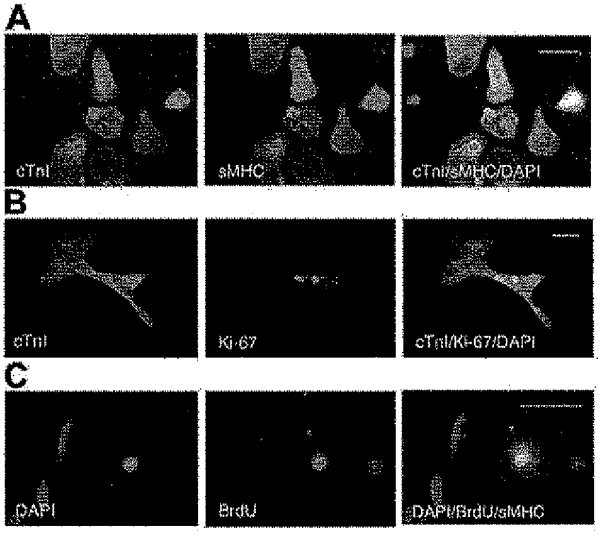

Figure 5. Characterization of Percoll-enriched cells. A, Immunostaining of H1 cells (passage 30) isolated at differentiation day 19, cultured for 2 days, and stained with antibodies against cTnI and sMHC. B, Immunostaining of H7 cells (passage 47) isolated at differentiation day 12, cultured for an additional 10 days, and stained with antibodies against cTnI and Ki-67. C, H7 cells (passage 37) isolated at differentiation day 29, cultured for additional 3 days, pulsed with BrdU, and stained with antibodies against sMHC and BrdU. Bar≈50 μm

SMA, suggesting that these cardiomyocytes may represent an early stage of cardiomyocytes.

To evaluate the proliferative capacity of these cells, cultures were analyzed for BrdU incorporation and Ki-67 expression. Ki-67 is a protein in active phases of the cell cycle (G1, S, G2, and mitosis) but not in resting G0 cells and therefore used to assess cell proliferation.[40,41] In this experiment, H7 cells (passage 37) at differentiation day 13 were dissociated and isolated by Percoll separation. Cells in fraction III and IV were replated, cultured for additional 2 days, and then pulse-labeled with BrdU for 24 hours. We found that 43±4% of the sMHC-positive cells expressed BrdU, indicating that these cardiac cells were in S phase of proliferation. Parallel cultures were Percoll-separated at differentiation day 29, cultured for additional 4 days, and assessed for BrdU incorporation and the presence of Ki-67. We found that 23±10% of sMHC-positive cells incorporated BrdU and 28±4% of sMHC-positive cells were positive for Ki-67. In sMHC-negative cells, 71±2% cells incorporated BrdU and 46±7% cells were positive for Ki67. Experiments using other cultures also indicated that a subset of cTnI-positive cells expressed Ki-67 (online Table 3). Figure 5 shows a representative image. These results indicate that some of the hES cell–derived cardiomyocytes were proliferating.

Discussion

The generation of functional cardiomyocytes from hES cells has several potential applications including myocardial repair through cell transplantation. Such an application has already been demonstrated in animal models using other sources of cells[4–11]; however, the plasticity of adult stem cells has been recently challenged.[42,43] The assumed capacity of transdifferentiation of the adult stem cells into other lineages in vivo might simply be a fusing with existing cell types rather than direct conversion. In addition, adult stem cells usually have limited proliferative capacity, whereas hES cells have extended replicative capacity.[26] Therefore, hES cell–derived cardiomyocytes may prove to be the best candidate population for cell therapy. This and other potential applications of hES cell–derived cardiomyocytes are, however, largely dependent on practical aspects of producing a sufficient amount of these cells.

Our data demonstrate that hES cells can effectively differentiate into functional cardiomyocytes. This conclusion is based on (1) the contractility of the differentiated cultures, (2) specific expression of multiple cardiac-associated molecular markers by the differentiated cells, and (3) appropriate response of these differentiated cells to cardioactive drugs. While this article was in preparation, Kehat et al[44] reported that cardiomyocytes can be produced from H9.2 hES cells. In the present study, we report that cardiomyocytes can be generated from multiple hES cell lines tested (H1, H7, H9, H9.1, and H9.2) and that, using the H9.2 cells, we observed a higher percentage of beating EBs (70% versus 8%) compared with the earlier report. The difference in the efficiency of cardiomyocyte differentiation may reflect differences in culture conditions of the undifferentiated hES cells, methods used for the dissociation of hES cells to generate EBs, the length of EB suspension culture, and/or the quality of serum used for differentiation. For example, we have been maintaining undifferentiated hES cells on MEF feeders or in feeder-free conditions using medium containing serum replacement. However, Kehat et al cultured cells on feeders in medium containing FBS. Different culture conditions could lead to a different status of the hES cells used for differentiation and may be influenced by the confluence of the culture and amount of undifferentiated versus spontaneously differentiated cells in the cell population. In our experiments, cells were harvested using 200 U/mL collagenase IV for 5 to 10 minutes and gently dissociated into cell clumps for EB formation. These clumps vary in size, but the majority contained ≈100 cells or more. However, Kehat et al treated cells with 1 mg/mL collagenase IV for 20 minutes, which resulted in smaller clumps containing 3 to 20 cells. In addition, we allowed the EBs to attach onto plates after culture in suspension for only 4 days instead of 10 days as described by Kehat et al.[44] It is likely that the microenvironment within the EB culture will influence the differentiation of the cell population.

We have found that cardiomyocyte differentiation can be significantly enhanced by treatment of cells with 5-aza-dC, a demethylation reagent. This might reflect a direct improvement of cardiomyocyte differentiation due to regulation of gene expression by demethylation. Alternatively, it might simply be a net effect from the lowered efficiency of hES cell differentiation into other cell types. Our observation underscores the importance of demethylation for hES cell differentiation into cardiomyocytes and perhaps other cell types as well.

We and others have previously reported that hES cells have different properties than mES cells, including surface marker expression and response to growth factors.[24–27] Consistent with this observation, hES cell cardiomyocyte differentiation is indeed quite different from cardiomyocyte differentiation from mES and mEC cells. We observed cardiomyocyte differentiation from hES cells maintained for 260 population doublings, although cardiomyocyte differentiation using late passages of mES cells has been difficult. Whereas DMSO and RA enhance mEC P19 or mES cell cardiogenesis,[28,29] these compounds did not show such an effect on hES cell cardiomyocyte differentiation. Although the exact mechanism is unclear, it is possible that cardiomyocyte differentiation from hES cells is controlled by different signaling pathways or a common pathway that is also regulated by species-specific modulators. The effects of RA we have observed are in contrast to those reported by Schuldiner et al,[45] who showed that RA treatment increased expression of cardiac α-actin in H9.1 clonal cell line. This difference may have resulted from several factors such as different cell lines or subclones, culture systems, differentiation protocols, and/or the assay endpoints used.

In addition, we have also demonstrated the enrichment of hES cell–derived cardiomyocytes by Percoll gradient separation and proliferation capacity of the enriched cells. These cells express appropriate cardiomyocyte-associated proteins. A subset of them appears to be proliferative as determined by BrdU incorporation or expression of Ki-67, suggesting that these cardiomyocytes represent an early stage of cells. This population may be a useful model for studying cell cycle regulation of the cardiomyocytes. It will be important to determine if this represents an expandable population of cells.

In summary, we have demonstrated that an enriched population of cardiomyocytes can be derived from hES cells. These hES cell–derived cardiomyocytes can now be tested for their ability to enhance cardiac function in preclinical animal models and for utility in drug discovery.

Acknowledgments

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EXHIBIT 7

A germ cell origin of embryonic stem cells?

Thomas P. Zwaka[1] and James A. Thomson[1,2,*]

[1]University of Wisconsin – Medical School and The National Primate Research Center, University of Wisconsin, Madison, WI 53715, USA
[2]WiCell Research Institute, Madison, WI 53715, USA
*Author for correspondence (e-mail: thomson@primate.wisc.edu)

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Summary

Because embryonic stem (ES) cells are generally derived by the culture of inner cell mass (ICM) cells, they are often assumed to be the equivalent of ICM cells. However, various evidence indicates that ICM cells transition to a different cell type during ES-cell derivation. Historically, ES cells have been believed to most closely resemble pluripotent primitive ectoderm cells derived directly from the ICM. However, differences between ES cells and primitive ectoderm cells have caused developmental biologists to question whether ES cells really have an in vivo equivalent, or whether their properties merely reflect their tissue culture environment. Here, we review recent evidence that the closest in vivo equivalent of an ES cell is an early germ cell.

Introduction

Embryonic stem (ES) cells are pluripotent (see Box 1) and can be expanded without limit in vitro (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). It is remarkable that permanent pluripotent stem cell lines can be derived from pre-implantation embryos at all, because, in vivo, pluripotent cells of the early mammalian embryo proliferate only briefly before becoming cells with a more restricted developmental potential. A few years after the initial derivation of mouse ES cells, it was suggested that they be called 'embryo-derived stem cells', a more precise term that would distinguish between these new pluripotent cell lines and cells within the embryo (Rossant and Papaioannou, 1984). However, this term was never adopted, and the extent to which these pluripotent stem cell lines represent any specific embryonic cell type or reflect their artificial tissue culture environment is still an open issue today – two decades later. Elucidating the origin of ES cells is of importance because it may help us to identify genes that are essential for the long-term maintenance of the pluripotent state. It could also assist with the derivation of ES cells from species whose ES cells have proved difficult to isolate. It will also help us to assess how accurately ES cell differentiation reflects events that normally occur in vivo. Here, we review the origin of ES cells, and explore recent evidence that ES cells are closely related to early germ cells.

The historical origins of ES cells: embryonal carcinoma cells

Historically, work with mouse teratocarcinomas paved the way for the derivation of ES cells. These germ cell tumors contain multiple differentiated tissues and undifferentiated stem cells, called embryonal carcinoma (EC) cells (Damjanov and Solter, 1974; Dixon and Moore, 1952; Kleinsmith and Pierce, 1964). Although teratocarcinomas had been known as medical curiosities for centuries (Wheeler, 1983), it was the discovery that male mice of strain 129 had a high incidence of testicular teratocarcinomas (Stevens and Little, 1954) that made these tumors more routinely amenable to experimental analysis. Because their growth is sustained by the persistent EC cell component (Stevens and Little, 1954), teratocarcinomas can be serially transplanted between mice. Eventually, conditions were developed that allowed the culture of EC cells in vitro, establishing them as an in vitro model of mammalian development (Kahan and Ephrussi, 1970).

As pluripotent cells of the intact early embryo proliferate for only a limited period of time, it was not initially obvious whether pluripotent cell lines could be established without undergoing malignant transformation. However, the transplantation of genital ridges or of egg-cylinder-stage embryos into ectopic sites, such as under the kidney capsule of adult mice, gave rise to teratocarcinomas at a high frequency in strains that did not spontaneously produce these tumors (Solter et al., 1970; Stevens, 1970a; Stevens, 1970b). These teratocarcinomas could be serially transplanted between adult mice, depending on whether the EC cell component persisted or differentiated (Solter et al., 1981). If the EC compartment disappears, the resulting tumor develops as a benign teratoma. Indeed, the malignant phenotype of EC cells often depends on the strain of the host mouse, and not on the tumor strain. EC cells injected into mouse blastocysts can contribute to either the normal tissues of the resulting chimera (Brinster, 1974) or, in some cases, to tumors (Rossant and McBurney, 1982). Because the ectopic transplantation of normal peri-implantation embryos can give rise to pluripotent cell lines, the direct derivation of pluripotent cell lines in vitro was attempted without the teratocarcinoma step. The culture conditions that were established to support mouse EC cells, including the use of feeder cell layers, were essentially those used to isolate mouse, and eventually human, ES cells (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998).

One indication that these early EC cell lines may be derived from germ cells (Solter et al., 1970; Stevens, 1967; Stevens, 1970a) came from mouse genital ridge-transplantation experiments. These experiments showed that genital ridges

Box 1. Glossary

Inner cell mass (ICM)
The second lineage of the early embryo that is located inside the blastocyst. It gives rise to all embryonic tissues.

Pluripotency
Refers to the unique ability of cells within the early embryo to differentiate into all cell types.

Primitive ectoderm
The remaining ICM tissue formed during the second differentiation event of embryonic development (also known as epiblast or embryonic ectoderm)

Primitive endoderm
An epithelial layer derived from cells that are in contact with the blastocyst cavity.

Trophectoderm
During the first differentiation event in mammalian development, morula cells segregate into two cell lineages: the first, the trophectoderm, forms the outer layer of the blastocyst. It eventually becomes part of the placenta.

effectively give rise to teratocarcinomas only in a narrow time window (E12.0-12.5). It is around this time that migratory germ cells start arriving in the genital ridge. In the egg cylinder-transplantation experiments, however, the origin of the EC cells was less clear. Primitive ectoderm seemed the most likely candidate for several reasons: because the potential to form teratocarcinomas is lost at the time when primitive ectoderm disappears at E8.5 (Damjanov et al., 1971); because EC cells have phenotypic similarities to primitive ectoderm cells in vivo (Diwan and Stevens, 1976); and because EC cells, when reintroduced into blastocysts, contribute to the same tissues as primitive ectoderm (Brinster, 1974). In addition, when analyzing the earliest stages of teratocarcinoma formation in 129/Sv mouse fetal gonads, Stevens observed clusters of polarized epithelial cells surrounding a central cavity that morphologically resembled primitive ectoderm cells (Stevens, 1983). He also observed that the formation of teratocarcinomas in ovaries included parthenogenic activation of the oocyte, the formation of blastocyst-like structures and the subsequent formation of structures that resembled early egg cylinders, which eventually became disorganized. Isolated transplanted primitive ectoderm itself gives rise to teratocarcinomas (Diwan and Stevens, 1976), but because early germ cells are just appearing at this stage, a germ cell origin cannot be completely ruled out by these experiments.

Are ES cells a tissue culture artifact?

ES cells clearly exhibit some properties that are not normally shown by cells of the intact embryo. For example, although ES cells retain properties of early embryonic cells in vitro, no pluripotent cell demonstrates long-term self-renewal in vivo. Embryonic cells, once brought into tissue culture, are exposed to numerous extrinsic signals to which they never would be exposed to in vivo. ES cells certainly adapt to selective tissue culture conditions and acquire novel functions that allow them to proliferate in an undifferentiated state indefinitely, and, because of this, ES cells are in some sense tissue culture artifacts (Buehr and Smith, 2003; Rossant, 2001; Smith, 2001).

As these changes are inevitable, the issue is not whether ES cells exhibit some properties that merely reflect their tissue culture environment, but rather whether they are most closely related to a specific in vivo cell type in the embryo, or if the influence of the culture environment is so dominant that it is impossible to relate ES cells to a single, in vivo cell type. We will certainly not completely resolve this issue here, but will re-explore the relationship of ES cells to specific early embryonic cell types.

Are ES cells most closely related to primitive ectoderm?

Although ES cell lines are generally derived from the culture of the ICM, some experiments suggest that ES cells more closely resemble cells from the primitive ectoderm. For example, isolated primitive ectoderm from the mouse gives rise to ES cell lines at a higher frequency than does isolated ICM. Moreover, the culture of primitive ectoderm allows the isolation of ES cell lines from mouse strains that have been previously refractory to ES cell isolation (Brook and Gardner, 1997). Indeed, ES cell lines can be derived from single, isolated, mouse primitive ectoderm cells, which is not possible with ICM cells (Gardner and Brook, 1997). Although these experiments suggest that ES cells are more closely related to primitive ectoderm than to ICM, they do not reveal whether ES cells more closely resemble primitive ectoderm or a cell derived from it in vitro.

A maximum of three individual cultured primitive ectoderm cells per embryo have been shown to give rise to ES cell colonies (Gardner and Brook, 1997). This low frequency could have been due to some variability in the potential of primitive ectoderm cells, to some variability in the environment in which they were placed or to damage caused by the dissociation of the primitive ectoderm into individual cells. However, by tracking the expression of the octamer-binding transcription factor 4 (*Oct4*) gene, a marker of pluripotency, in intact cultured ICM/epiblast cells, it was shown that *Oct4* expression was maintained in only a small proportion of outgrowing cells (Buehr et al., 2003), which also suggests that only a minority of primitive ectoderm cells can transit to a new stable, proliferative pluripotent state, and, subsequently, be expanded as ES cells. These results could be due to a requirement for a relatively rare intrinsic or extrinsic stochastic event, or to an inherent heterogeneity of the primitive ectodermal cell population. Recent data indicate that even the earliest ICM is heterogeneous and consists of a mixture of cells that express either *Oct4* or *Gata6* (Rossant et al., 2003), and a similar later heterogeneity could account for the fact that only a minority of primitive ectoderm cells generally give rise to ES cells in culture.

Established mouse ES cell lines express some specific markers of primitive ectoderm at a very low level, if at all (Table 1), such as fibroblast growth factor 5 (*Fgf5*) (Haub and Goldfarb, 1991; Hebert et al., 1991; Rathjen et al., 1999). Culture conditions have been established that convert mouse ES cells into early primitive ectoderm-like cells that express both *Fgf5* and *Oct4* (Rathjen et al., 1999), but these cells fail to form chimeras when injected into mouse blastocysts. Taken together, these results suggest that ES cells are most closely

Table 1. Marker genes expressed in embryonic stem cell (ES), early germ (EGC) and later germ cells (LGC), in the inner cell mass (ICM) and in the primitive ectoderm (PE)*

Gene	Species	ES	EGC	LGC	ICM	PE
Pou5f1 (Pesce and Scholer, 2001)	M	+	+	+	+	+
Nanog (Chambers et al., 2003)	M	+	+	+	+	+
Dppa3 (Saitou et al., 2002)	M	+	+	+	+	+
Ifitm3 (Saitou et al., 2002)	M	+	+	+	+	+
Kit (Horie et al., 1991)	M	+	+	+	–	N/D
DAZL (Clark et al., 2004)	H	+	+	+	–	N/D
Ddx4 (Toyooka et al., 2003)	M	–	–	+	–	–
Akp2 (Chiquoine, 1954)	M	+	+	+	+	+
Zfp42 (Rogers et al., 1991)	M	+	N/D	N/D	+	–
Fgf5 (Haub and Goldfarb, 1991; Hebert et al., 1991)	M	–	N/D	N/D	–	+
Gbx1 (Chapman et al., 1997)	M	+	N/D	N/D	+	–

*Data are based on mouse (M) and human (H) studies, some are preliminary.
+ denotes expression at that developmental stage, – denotes the gene is not expressed
N/D, not done.

related to a subpopulation of primitive ectoderm cells, or to a close derivative of primitive ectoderm cells.

One of the curious species-specific differences between human and mouse ES cells is that human ES cells give rise to trophoblast cells at a high efficiency (Xu et al., 2002), but mouse ES cells do not (Beddington and Robertson, 1989). In the intact mouse embryo, the last cells capable of giving rise to trophoblast cells are early ICM cells, so the failure of mouse ES cells to differentiate into trophoblast is good evidence that they are not the equivalent of early ICM cells (Brook and Gardner, 1997). The differentiation of human ES cells to trophoblast could be explained if they are related to an earlier cell type than mouse ES cells, or if the specification of the trophoblast lineage occurs differently in human embryos. However, a third possibility is that ES cells represent a different cell type altogether. It is therefore worthwhile examining the relationship between ES cells and germ cells.

Germ cells and the primitive ectoderm

In elegant, clonal-fate mapping studies in the mouse (Lawson and Hage, 1994), germ cells were shown to arise from a founder population in the E6.0-6.5 proximal epiblast adjacent to the extra-embryonic ectoderm. These founder cells then pass through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. By E7.25, a distinct cluster of ~45 tissue non-specific, alkaline phosphatase (Tnap)-positive germ cells is present at the base of the allantois (Fig. 1) (Ginsburg et al., 1990). The E6.5 distal epiblast, which would not normally contribute to germ cells, will contribute to germ cells if transplanted to a proximal location (Tam and Zhou, 1996), which demonstrates that location and inductive signals, rather than germ plasm determinants, are responsible for the specification of germ cells in mice (Extavour and Akam, 2003). This flexibility suggests that cultured primitive ectoderm cells could spontaneously give rise to early germ cells in culture.

Bone morphogenetic protein 4 (Bmp4) (Lawson et al., 1999) and Bmp8b (Ying et al., 2000) are required for the formation of the proximal posterior extra-embryonic region that gives rise to primordial germ cells (PGCs) and to cells of the allantois in the mouse. The addition of Bmp4 and Bmp8b to distal mouse epiblast cultures increases the formation of cells strongly positive for Tnap (Ying et al., 2001), a marker shared by early germ cells and ES cells. These Tnap-positive cells were interpreted as being germ cells in this study. Recently, BMP signaling has been shown to be important for the self-renewal of mouse ES cells (Ying et al., 2003), and although BMPs are involved in many differentiation decisions in the early embryo, these results do further hint at a relationship between ES cells and early germ cells.

Similarities between germ cells and ES cells

In mice, PGCs migrate and proliferate until ~25,000 are present in the genital ridge at E13.0 (Tam and Snow, 1981). Pluripotent cell lines from pre- and post-migratory (Resnick et al., 1992; Matsui et al., 1992; Shamblott et al., 1998), as well as from migratory (Durcova-Hills et al., 2001), germ cells have been isolated, and these cell lines are termed embryonic germ (EG) cells to distinguish their origin. Mouse EG cell lines are remarkably similar to mouse ES cell lines (Donovan and de Miguel, 2003). During germ cell migration and maturation, however, the somatic status of imprinted genes is progressively erased (Yamazaki et al., 2003), and EG cells isolated at various stages of migration retain some of these differences, such as the reduced methylation of many imprinted genes, including *H19* and *Snrpn* (Hajkova et al., 2002). The analysis of mouse PGCs at E10.5 suggests that methylation erasure has already begun by this time, as supported by studies of the expression of imprinted genes (Yamazaki et al., 2003). This study showed that imprinted genes, such as *H19* and *Snrpn*, exhibit imprinted (somatic) expression patterns in E9.5 PGCs, but by E10.5 have switched to a bi-allelic mode of expression (Yamazaki et al., 2003). Because the genes expressed in ES cells exhibit somatic imprinting patterns (Geijsen et al., 2004), their change in imprinting status suggests that if ES cells are derived from germ cells, this derivation must occur before E9.5.

There is a paucity of known molecular markers that distinguish early germ cells from other pluripotent cells of the early embryo. One marker, Tnap, is strongly expressed by early germ cells and by ES cells, but is weakly expressed by the epiblast and other surrounding embryonic cells (Chiquoine, 1954; Ginsburg et al., 1990). Two new markers for early germ cells, fragilis (Ifitm3 – Mouse Genome Informatics) and Dppa3 (also know as stella or PGC7), have recently been identified that allow the better separation of early germ cell precursors from their differentiated neighboring cells (Saitou et al., 2002).

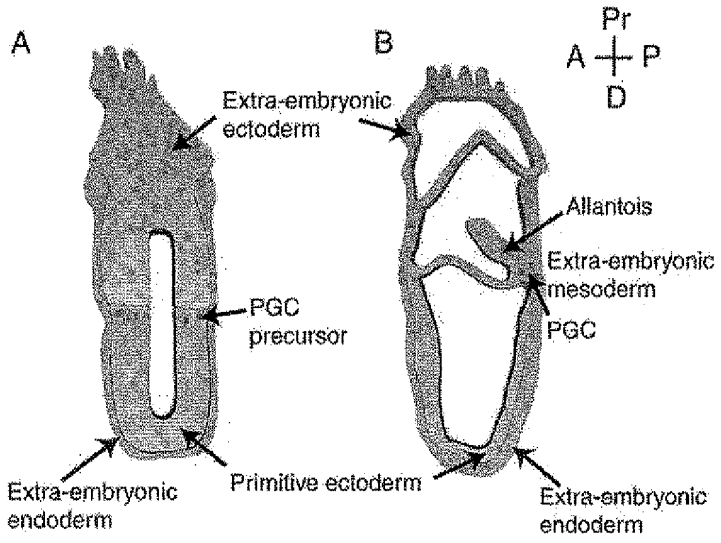

Fig. 1. Early development of the mouse embryo. (A) Six days after fertilization (E6.25), the mouse embryo consists of three layers. The inner cell mass (ICM) cells that are in contact with the blastocyst cavity differentiate into an epithelial layer called the extra-embryonic (primitive) endoderm. The rest of the ICM becomes the epiblast (primitive ectoderm). Primordial germ cells (PGCs, red dots) arise from a cell population in the proximal epiblast adjacent to the extra-embryonic ectoderm. These cells then pass through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. (B) By E7.25, a distinct cluster of ~45 tissue non-specific, alkaline phosphatase (Tnap)-positive PGCs is present at the base of the allantois within the extra-embryonic mesoderm (red dots). Once these PGCs are specified, they begin to migrate to the future gonadal anlagen. A, anterior; P, posterior; Pr, proximal; D, distal.

Dppa3 is expressed in pre-implantation embryos and in germ cells (Sato et al., 2002) and has recently been reported to have a role as a maternal transcript in preimplantation embryonic development (Bortvin et al., 2004). Dppa3-positive cells show increased expression of fragilis and remain positive for *Tnap* (*Akp2* – Mouse Genome Informatics) and *Oct4* (Saitou et al., 2002). Once Dppa3-positive PGCs start to migrate, they begin to express additional markers, such as steel factor receptor, followed by markers of more mature germ cells, such as murine vasa homolog (MVH; Ddx4 – Mouse Genome Informatics) (Saitou et al., 2002).

Several recent reports describing the differentiation of mouse ES cells into cells that express markers of mature male and female germ cells (Geijsen et al., 2004; Hubner et al., 2003; Toyooka et al., 2003) are important for our understanding of the origin of ES cells. In each of these reports, germ cell markers were expressed by ES cells themselves, including those, such as Dppa3, that help distinguish germ cells from primitive ectoderm (Table 1). Only the expression of more mature germ cell markers (such as MVH) enabled in vitro-derived germ cells to be distinguished from ES cells themselves. In one study that examined the differentiation of human ES cells into germ cells (Clark et al., 2004), the expression of each of eight genes that are characteristic of early germ cells was detected in human ES cells, but the expression of each of six genes that are characteristic of later germ cells was not detected, strongly suggesting that the expression of the early germ cell-genes was not merely a result of the broadly 'leaky' transcription that is often attributed to ES cells. Using immunocytochemistry, it was also shown that most individual human ES cells in a population express the early germ cell markers stella related (STELLAR) and deleted in azoospermia-like (DAZL), indicating that a minor subset of randomly differentiating cells in a mixed population is not responsible for the expression of germ cell markers in ES cell cultures. Importantly, it was also shown that at least one germ cell-specific gene, *DAZL*, was expressed by human ES cells but not by human ICM. The existing gene expression data, then, are consistent with the idea that the closest in vivo equivalent to ES cells is not the ICM or primitive ectoderm, but an early germ cell.

Some of the properties of ES cells, however, suggest that they are not merely the equivalent of early germ cells. For example, the earliest PGCs do not self-renew for prolonged periods of time, but instead begin a series of maturation steps, beginning with germ cell migration and ending in the highly specialized development of sperm or egg (Wylie, 1999). Although ES cells can differentiate into more mature germ cells in vitro, they do so relatively inefficiently. Indeed, the ability to colonize the germline of chimeras is one of the most easily lost properties of ES cells. If ES cells most closely represent early germ cells, it is unclear why they are not better at giving rise to more mature germ cells. In addition, isolated PGCs have never been demonstrated to contribute to chimeras when injected into blastocysts, so an exact equivalence to ES cells is unlikely.

Because a comprehensive and comparative analysis of the transcriptomes of isolated ICM, primitive ectoderm and early germ cells has not yet been reported, it is not yet clear how much the particular repertoire of genes expressed by ES cells represents an early germ cell, another specific in vivo cell type, a response to the tissue culture environment, or a combination of all three. If the ICM and primitive ectoderm are inherently heterogeneous, transcriptome analysis may need to be carried out at the single-cell level to ultimately understand these relationships. However, at the moment, the greatest concordance of known markers appears to be between ES cells and early germ cells.

Conclusions

We hypothesize that ES, EC and EG cells represent a family of related pluripotent cell lines, whose common properties reflect a common origin from germ cells (Fig. 2). Although a more detailed transcriptional analysis could ultimately refute the proposed relationship between ES cells and early germ cells, we hope this idea will at least help to stimulate a healthy

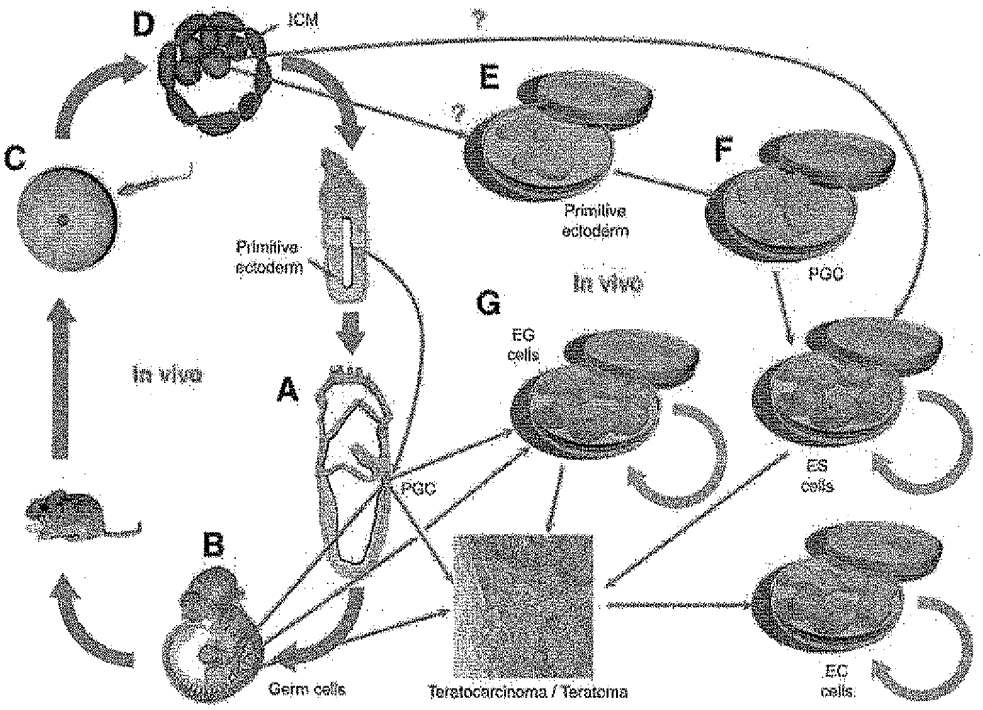

Fig. 2. Germ cell propagation in vitro and in vivo. (A-C) The germ cell cycle in the mouse. (A) Primordial germ cells (PGCs) appear at E7.25 as a small group of cells (red) in the extra-embryonic mesoderm. (B) After E8.5, PGCs start to migrate to the gonadal anlagen and contribute (C) during puberty to oocytes or sperm. (D) Embryonic stem (ES) cells are in vitro derivatives of inner cell mass (ICM) cells. (E,F) The formation of ES cells occurs either directly from the ICM/primitive ectoderm (E) or according to our hypothesis, through in vitro differentiation of ICM outgrowth into primitive ectoderm, then into extra-embryonic mesoderm and finally into PGCs (F). (G) PGCs that form in vivo (A-C) give rise to embryonic germ (EG) cells in vitro. Germ cells, PGCs, ES and EG cells are all capable of forming teratomas and teratocarcinomas. ES and EG cells can reintegrate into the normal embryo after injection into the blastocyst (not shown). Circular green arrows denote unlimited self-renewal.

re-evaluation of what is actually being studied when ES cells differentiate in vitro.

What is the relevance of a putative close relationship between ES cells and early germ cells? One prediction of this hypothesis is that at least some of the germ cell-specific genes expressed by ES cells, and not by primitive ectoderm cells, are essential for the long-term maintenance of the pluripotent state. If true, then it should be possible to generate knockout mice to identify genes that are essential for the specification or maintenance of PGCs, which are also essential for the derivation of ES cell. A related prediction of the hypothesis is that genes that are responsible for increasing susceptibility to spontaneous germ cell tumors should increase the efficiency of ES cell derivation. It is interesting, for example, that in species where teratocarcinomas occur at a clinically significant frequency, such as in mouse and human, ES cells have been successfully derived, whereas in species where teratocarcinomas are exceedingly rare, such as the rat, ES cells have proven difficult to derive. Understanding basic species differences in the specification or maintenance of early germ cells could allow the derivation of ES cells from species that have been hitherto resistant to the isolation of ES cells, such as the rat (Buehr et al., 2003).

Another implication of our hypothesis is that when looking for evolutionary clues to understand the pluripotent state, the comparative germ cell literature will be the most instructive. In a species such as the zebrafish, which has a germ plasm that strictly separates germ cells from somatic cells, it makes sense that pluripotent cell lines that can contribute to the germline in chimeras (Ma et al., 2001) would have to be derived from germ line-lineage cells.

Another prediction arising from the hypothesis that ES cells most closely represent early germ cells is that the very earliest events of ES cell differentiation into somatic and extra-embryonic lineages will not accurately reflect events that normally occur in vivo. The idea that ES cells represent an in vitro equivalent to the ICM, however, is firmly entrenched and continues to strongly influence our thinking about these cells. When examining the differentiation of ES cells in vitro, the pervasive mental image is of a forward progression that recapitulates normal embryonic events. For example, one thinks of ICM cells progressing to primitive ectoderm cells, then to neural ectoderm cells, and finally to more specialized neural cell types. If ES cells most closely represent early germ cells, this mental image needs revision, as the earliest transition would appear to be more 'lateral' or even 'backward' than 'forward'. It will be illuminating to define each of the distinct transitions that ES cells can make in a single step and to determine how much these initial transitions resemble in vivo or artificial differentiation. If ES cells really represent early germ cells, the initial events in differentiation would be expected to be transitions that do not normally occur in intact

embryos, except, perhaps, when the transition is to more mature germ cells.

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